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DROUGHT INDUCED MORPHOLOGICAL AND COMPOSITIONAL CHANGES IN CREEPING BENTGRASS (*AGROSTIS STOLONIFERA* VAR. L. *PALUSTRIS*) CUTICLE AS IT INFLUENCES FOLIAR NITROGEN ABSORPTION

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ABSTRACT

Creeping bentgrass is the most popular turfgrass species on golf course putting greens throughout the world due to its fine texture, recuperative ability and dense growth habit. Drought stress is an environmental induced condition that is common to turfgrasses. Foliar fertilization is a common maintenance practice that is conducted and utilized on golf course putting greens due to the reduced total input, quick response and reduced environmental impact. The cuticle of creeping bentgrass creates a hydrophobic barrier that foliar applied fertilizers need to penetrate to be used by the plant. The cuticle morphology and composition is subject to change due to the environment. Therefore, the primary objectives of this thesis were to determine how the cuticle of creeping bentgrass was affected by drought stress, understand the influence of the cuticle on foliar fertilization absorption, and investigate methods to aid foliar applications.

Due to the limited literature on creeping bentgrass cuticle layer and methodology of studying the cuticle layer of grasses, a preliminary growth room study was conducted. Creeping bentgrass plugs were harvested and transplanted into a growth room where treatments were conducted. Treatments included a control with 100% ET returned and two drought treatments, where 50% and 25% ET was returned. Cuticle morphological and compositional changes were studied. Results revealed, 1-hexacosanol, comprising approximately 88% of the entire cuticle of creeping bentgrass. The remaining 12% was comprised of fatty acids, alkanes, an aldehyde and a five unknown compounds. Total wax load showed a pattern of increasing due to the drought stress. Cuticle crystalloid density increased significantly with drought stress. ^{15}N-labeled urea was applied foliarly and % recovery was determined to evaluate the effect of drought on foliar uptake. Percent ^{15}N recovery was significantly reduced in the two drought treatments compared to the control. The increase in total wax was negatively correlated with %^{15}N recovery. Results suggest cuticle morphology and quantity may limit foliar fertilizer absorption.

A second study was conducted investigating drought stress on creeping bentgrass cuticle and foliar absorption. Treatments included control and drought where 100% or 50% ET was returned daily for 10d. Foliar absorption was studied with ^{15}N-labeled urea applied with or without surfactant addition. Cuticle morphology and compositional changes were studied along with % ^{15}N recovery. Total wax and crystalloid density increased significantly due to drought treatments. This was caused mainly by an increase in primary alcohols and fatty acids. Percent ^{15}N recovery was affected by irrigation treatment and surfactant addition. The results presented suggest that the surfactant addition allows for improved penetration or adherence to cuticles influenced by drought stress.

Genetically modified plants can be engineered to tolerant environmental stresses, and could prove beneficial to regular maintenance practices. Transgenic creeping bentgrass was developed at Clemson University using the overexpression of *Arabidopsis* vacuolar H^+-pyrophosphatase (AVP1) gene. Wild-type (WT) and transgenic (TG) cuticles were studied for differences in morphology and composition under control and drought treatments. The TG cuticle morphology was similar to the WT, but small differences were seen. Crystalloid density increased due to drought in the WT but not in the TG. Irrigation treatment had no significant effect on total cuticle wax for either WT

or TG. Interestingly, the TG cuticle wax load was significantly higher (>1 µg cm^{-2}) than the WT cuticle wax load. This was due to a larger amount of primary alcohols and fatty acids in the TG cuticle. Results suggest that the TG cuticle provides a less rough surface that would not influence a foliar applied solution, especially under drought stress where crystalloid density is not affected.

Fractal analysis was used to study and classify the crystalloid shape of WT and TG creeping bentgrass. The analysis was used to related cuticle chemical composition data, crystalloid shape and the fractal dimension. It was revealed that there was a significant difference in fractal dimension for the TG and WT crystalloids. The change in fractal dimension was thought to be the TG cuticle preparing itself for stress and therefore making its cuticle more hydrophobic to create a better protection barrier.

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CHAPTER ONE

INTRODUCTION

Creeping bentgrass (*Agrostis stolonifera* var. L. palustris) is the most widely used cool-season turfgrass for golf course putting greens. Frequent applications of foliar absorbed fertilizer are a regular practice on putting greens for proper growth and green performance. Foliar fertilization provides many benefits including: a reduction in leaching of nutrients, faster absorption, a more uniform growth response, and can be beneficial when nutrient availability in the soil is low due to pH, soil structure, and salinity or drought events (Marschner, 1995). Although foliar fertilization is commonly practiced, the knowledge of the mechanisms of absorption and uptake of foliar applied nutrients is lacking. The study of plant cuticles has been active over the past century, but unfortunately little is known about the cuticle layer of turfgrasses. Although the cuticle protects the plant from uncontrolled water loss, it also acts as a barrier to foliar applied solutions.

The plant cuticle is a continuous extracellular membrane which covers the primary above ground organs of all lower and higher land plants (Koch and Ensikat, 2008). Cuticle ultrastructure varies greatly between plant species, organ types, and their developmental stages. Though the composition is variable, cuticles are mainly comprised of two materials. The first being cutin, an insoluble cross-linked polymer of hydroxyl and hydroxyepoxy-fatty acids, most commonly of C_{16} and C_{18} chain lengths (Holloway, 1982). The second main component of plant cuticles are lipids called “waxes”. These waxes are made up of various long chain hydrocarbon compounds such as primary and secondary alcohols, alkanes, aldehydes, ketones, and fatty acids (Riederer and Muller, 2006). Plant waxes that are integrated or embedded in the cuticle are known as "intracuticular waxes", and waxes that are superimposed onto the cuticle are referred to "epicuticular waxes" (Koch and Ensikat, 2008).

The major importance of the cuticle layer can easily be seen because it represents one of the largest interfaces between the plant body and the atmosphere (Riederer and Schreiber, 1995). This protective layer is involved in several major functions of the plant. The first is the cuticle and stomata act together and control water loss through transpiration. This barrier impedes the loss of ions and polar solutes, while also hindering solutes from entering the plant (Riederer and Muller, 2006). Studies involving scanning electron microscopy (SEM) have revealed that most epicuticular waxes form 3-D structures termed crystalloids. These crystalloids vary in shape and size depending on plant taxa (Barthlott et al., 1998). These crystalloids add to surface roughness which has been shown to have a direct effect on the contact angle of a water droplet (Yoshimitsu et al., 2002). Contact angles can be related to water retention capacity, which is significant for foliar deposition and uptake of foliar applied nutrients (Cape, 1996). It has been shown that plant micro- and nanostructures, such as, cellular protrusions, cuticular folding and epicuticular waxes reduce the contact area between water and the plant surface (Barel et al., 2006).

Effects of the environment and plant age have an extreme influence on the cuticle layer of the plant. Shepherd and Griffiths (2006) reported that temperature, light intensity and humidity influenced wax morphology, although it may be difficult to distinguish between the effects of each because they tend to occur simultaneously. For instance, tubular crystal forms have been shown to transform into more compact planar stable forms when subjected to heat (Baker, 1974). Giese (1975) found that leaf-surface wax density and deposition rates increased 2.5 times on barely (*Hordeum vulgare* L.) plants in light compared to plants in the dark. High humidity and low light intensity, both reduce wax accumulation making plants more susceptible to desiccation (Shepherd and Griffiths, 2006). Lastly, Bondada (1997) revealed that as the leaf aged in cotton (*Gossypium hirsutum* L.) plants the cuticle wax content increased, which decreased the absorption of ^{15}N applied to the leaves.

Abiotic stress can have a significant impact on the morphology and wax thickness of the plant cuticle layer. In response to water stress wax deposition often occurs very rapidly, increases in cuticle wax and thickness due to drought have been well documented (Bondada et al, 1996; Cameron et al., 2006; Kim et al., 2007a; Kosma et al., 2009). Jordan et al. (2001) showed an increase in wax production by ornamental trees irrigated with treated sewage effluent water. Cold stress was studied in maize (*Zea mays* L.) and revealed exposure to this stress reduced cuticular wax by 29%, thus increasing wettability and herbicide retention (Gauvrit and Gaillardon, 1991). Under stress the chemical composition of the waxes may change, but for most species this change is more quantitative rather than qualitative. For instance, there might be an increase in wax production due to drought or higher light intensities, instead of the omission of a single wax compound (Kock and Ensikat, 2008). A recent study revealed a decrease in nitrogen absorption in September compared to May in creeping bentgrass, which authors attributed to changes in the leaf cuticle through the stressful summer months making the leaves more hydrophobic and less receptive to nutrient absorption (Stiegler et al., 2010).

The cuticle layer provides a protective barrier to the plant, while also hindering the uptake of foliar applied nutrients. Previous research demonstrates the cuticle layer of plants can change significantly under different environmental conditions and abiotic stress. With foliar applied nutrients and chemicals a staple in many turfgrass maintenance programs and with the limited knowledge of foliar absorption the need for research is growing. The objectives of the studies were to provide knowledge on the morphological and compositional analysis in creeping bentgrass cuticle layer, and how the plant adapts the cuticle to drought stress. Also, foliar fertilization was analyzed to study the effects of the cuticle and how it might influence absorption. Next, the addition of a surfactant to the nutrient solution was investigated to determine if any adverse effects seen in the cuticle can be overcome. Lastly, the cuticle of creeping bentgrass was compared to a genetically modified creeping bentgrass to determine if there were possible benefits of the transgenic grass compared to the wild-type. This research has improved our understanding of the main barrier to foliar absorption, the cuticle layer, which will ultimately minimize cost, reduce environmental impact and improve plant health.

CHAPTER TWO

LITERATURE REVIEW

Creeping Bentgrass

Creeping bentgrass is the most widely used cool season grass used for golf course putting greens (Turgeon, 2012). It's native to central Europe where the climate is cool and moist. First introduced as a forage grass to North America, creeping bentgrass has since been cultivated to the closely mown golf course turfgrass. Although creeping bentgrass is adapted to the cool, humid climates in the Northern United States, its use spreads to other climatic regions as golf course putting greens (McCarty, 2011).

The common name creeping bentgrass comes from the stolons that develop atop the soil surface, where new roots and shoots form at the internodes. The plant description of creeping bentgrass is as follows: rolled vernation; sheaths round, glabrous, split with overlapping, hyaline margins; has a membranous ligule, 1-2 mm long from acute to oblong and may be notched; collar narrow to medium broad; 2-3 mm, flat blades; stems erect or ascending from a spreading decumbent base with long, slender stolons rooting at the nodes; a narrow, dense inflorescence pale purple in color (Beard, 1973; Turgeon, 2012).

Creeping bentgrass is adapted to fertile, fine textured soils of moderate acidity and good water holding capacity, however it has a tolerance for a wide range of soil types. A slightly acidic soil pH range from 5.5 to 6.5 is ideal, this species has shown better tolerance to saline conditions and flooding compared to other cool-season turfgrasses (Beard, 1973).

This grass is ideal for putting greens because of its fine texture, dense canopy, ability to tolerate low mowing heights and it's very aesthetically pleasing (Emmons, 2008). There have been a wide range of cultivars used throughout the years, with the most common being 'Penncross'. Recently, the development of new cultivars such as, 'Penn A-1', 'A-2', 'A-4','G-1', 'G-2', and 'G-6', can tolerate low mowing heights of 3.2 mm and lower, while sustaining very high shoot density (Beard, 2002). Because of creeping bentgrass's aggressive growth habit, there is significant thatch buildup and must be managed properly. The new cultivars need to be aerated, topdressed and cultivated more frequently to account for the more aggressive growth habit (McCarty, 2011).

Creeping bentgrass is susceptible to a variety of biotic and abiotic stresses (Dernoeden, 2013). Diseases cause a major problem for managers, as creeping bentgrasses are susceptible to a wide range of diseases. A preventative fungicide program is needed in regions where problems are anticipated (Beard, 1973). Abiotic stresses can become severe problems, especially throughout the hot humid summer months and in the regions outside its normal adaptation range. Excellent cold tolerance is seen in creeping bentgrass cultivars, but winter desiccation will develop when thatch accumulates and crowns and roots are above the soil surface (Fry and Huang, 2004). During the hot, humid months of the summer, the growth and development decreases, this results in a very shallow root system. The decrease in roots makes the grass even more susceptible to drought, traffic and diseases. For the increase in susceptibility there could be a need to alter the microclimate, such as, soil preparation, irrigation, air circulation, shade and other parameters (McCarty, 2011).

Drought Plant Responses

One of the most common and most limiting factors that affect plants' growth and development is reduced water. Recently, very severe droughts have been seen in the southeast and across the rest of the country, causing major problems in plant productivity. Because water is such a vital component in the plant cycle and regular processes, drought stress has been studied extensively and many of the mechanisms the plant utilizes to defend itself from this stress have been identified.

Taiz and Zeiger (2010) defines drought as a period of insufficient precipitation that results in plant water deficit. The mechanisms involved in the defense of plants to water stress are in three main categories: tolerance, avoidance and escape (Tuberosa et al., 2003). Alam (1994) states that if water is not available, fertilizer applications are useless, this is because water stress has a wide range of effects on morphological and physiological processes of plants.

One of the first responses to water deficit in the soil is cellular dehydration, this loss in cell water results in loss of turgor pressure and cell volume. After the reduction in turgor, the apoplast water potential becomes more negative than the symplast. As a result of reductions in cell turgor, cell expansion is reduced, which results in a decrease in growth rate. Another secondary effect of water loss is that ions in the cell become more concentrated and could become toxic (Taiz and Zeiger, 2010).

Another quick response that plants have when they incur reduced water is stomatal aperture. To avoid excessive water loss or hinder the uptake of gaseous pollutants, stomatal opening is regulated by the uptake and loss of water in guard cells. Not only does water loss in the guard cells produce stomatal closure, but it is almost always regulated by the presence of abscisic acid (ABA). ABA is an excellent stress marker, because it has been shown to increase under slight dehydration in root tips (Sharp and Davies, 1989). This plant hormone controls solute loss in guard cells, which is caused by the loss of water in the cell. Stomatal aperture regulation is just one of the number of processes the plant utilizes to control excessive water loss.

The stomata are also very involved in cooling the plant from high temperatures and could possibly damage the plant. Water is evaporated through transpiration from the stomata, taking with it heat from the leaf. If water is not available under drought conditions or stomates have closed, there are other ways the plant can regulate its temperature and avoid excessive energy absorption. This is leaf orientation. In order to protect themselves from overheating some plant species are able to orient their leaves away from the sun to reduce temperature and excessive energy capture. This was shown in soybean as a response to drought and photosynthetic photon flux (Kao and Forseth, 1992). Leaf wilting and leaf rolling are two other examples of secondary effects that make occur due to water stress as the plant tries to protect itself.

As mentioned earlier, reductions in turgor pressure is one of the first plant responses to drought stress. Because cell expansion is a result of turgor pressure, one can see how leaf area can be affected by reduced water stress. Cell cycles can be slowed or ceased by signaling resulting from reduced water which affects cell division and expansion, and can reduce the leaf area (Anami et al., 2009).

Another function that plants use to defend against excessive water loss during drought conditions is osmotic adjustment. Plants can increase their solute concentration inside cells so that they have a more negative water potential. This allows plants to create a potential more negative than the drying soil (Delauney and Verma, 1993). Although this can help alleviate one problem another may arise if too many ions accumulate. For instance, sodium and chloride are essential to plant growth, but at high concentrations can become toxic plants.

It has been shown the plant adjusts its' cuticle in response to water deficit to reduce transpiration and possibly reflect radiation. This topic will be covered in detail later in the review.

The Plant Cuticle

The plant cuticle is a continuous extracellular membrane which covers the primary above ground organs of lower and higher land plants. Only the majority of roots, some mosses and secondary plant tissue do not have this protective layer (Koch and Ensikat, 2008). This barrier has been studied rather extensively over the last century, with many questions being answer through diligent research, but unfortunately there are still gaps in the information and mysteries still need to be solved.

The cuticle being the main interface between plants and their environment, it must be able to provide the plant with many benefits and functions. The first and most notable function the cuticle gives to the plant is the protection against uncontrolled water loss to the atmosphere through transpiration (Burghardt and Riederer, 2006) (Fig. 2.1.). This barrier acts together with stomata to limit water loss, especially in unfavorable weather conditions such as drought and will undergo developmental changes when sensing water stress, and will be discussed in more detail later in this review. The second major function of the cuticle is closely related to control of water loss, it is the control of loss and uptake of polar solutes. The ion and polar organic solute rich, apoplastic solution would diffuse out of the plant into the atmosphere without the protective layer (Riederer and Muller, 2006). Although the importance of the cuticle can be seen with this function it also becomes a problem when discussing foliar applied nutrient solutions, because it becomes a barrier for the beneficial solution applied. The cuticle also serves the plant by controlling the exchange of gases and vapors into and out of the plant. This does not just mean water vapor, but also consists of oxygen, carbon dioxide, air pollutants and volatile organic compounds (Riederer and Muller, 2006).

Another function of the cuticle layer for the plant involves the repellent nature of the cuticle to water and particles. With the repellency of water, the cuticle provides the plant with protection from leaching of ions and solutes from the interior of the plant, and also protects the plant from colonization of microbes or fungi that could enter the plant in the aqueous solution that would be present if not repellent to water (Riederer and Muller, 2006). A self-cleaning mechanism termed the Lotus effect allows water droplets to form, when the droplets roll off dust, soot, spores, and microbes can be removed from the plant surface (Bargel et al., 2006). This self-cleaning aspect of the cuticle could be one of the most important functions of epicuticular waxes (Barthlott, 1990).

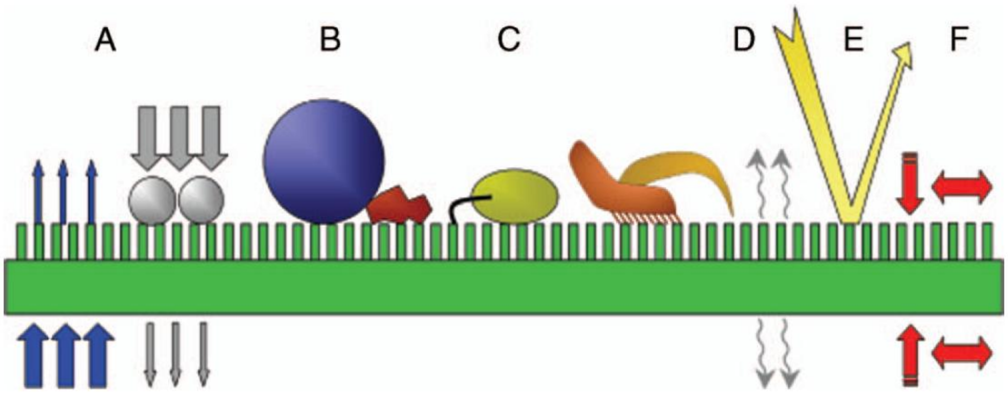

Figure 2.1: Diagram revealing the various functions of the plant cuticle layer. A: Limits uncontrolled water loss, leaching of solutes and foliar absorption. B: Water repellency, control water on surface. C: Anti-adhesive: self-cleaning properties. D: Signaling between host and pathogen. E: Reflection of UV-radiation. F: Resistance to mechanical damage. (Bargel et al., 2006)

Another function that has received some interest by researchers is the ability of the cuticle to reflect and scatter light hitting the plant surface. The cuticular surface topography, which will be discussed later in detail, can serve to reflect light much in the same way leaf hairs do on certain plant species (Shepherd and Griffiths, 2006). There have been numerous studies that investigated the reflectance capability of leaves and how the leaves affect photosynthesis and leaf protection. Cameron (1970) studying *Eucalyptus* species showed that reflectance decreased and photosynthesis increased when wax was removed from waxy-leaves, but the effect in non-waxy species was small. In a more recent studied using *Eucalyptus* and *Kalanchoe* species similar results were revealed; one that waxy leaves reflect more light than various species with leaf hairs, and that when the wax was removed reflectance decreased and absorption increased, also it was determined that non-waxy leaves showed little change in reflection when waxes were removed (Holmes and Keiller, 2002). It has been suggested that another function of light reflection from glaucous leaves could aid in illuminating lower leaves in the canopy, benefiting plants that have large leaf areas (Eller, 1979). Shepherd and Griffiths (2006) also mention that light reflectance by the leaf surfaces can help reduce leaf temperatures, reducing transpiration by lowering the vapor pressure difference between tissue and air.

Biotic interactions with the plant occur with and through the cuticle layer. From the microscopic scale with bacteria, yeasts and fungi, all the way up to herbivore behavior have been shown to be affected by the cuticle layer (Leveau, 2006; Carver and Gurr, 2006; Muller, 2006). The epicuticular waxes also provide protection from mechanical stress or damage like wind abrasion and rain droplets.

The chemical composition of the cuticle layer is a diverse and ever changing group of compounds. Besides for the cutin intermingled throughout the cuticle, its second major chemical component is wax. There has been a considerable amount of research, investigating analytical procedures, qualitative and quantitative wax composition, and epicuticular wax components. Jetter et al. (2006) provided a thorough review of different techniques and equipment that has been used to identify and study the cuticle of plants and its composition. The cuticular lipids are a mixture of aliphatic and aromatic components, and depending on species, the mixture is made up of various combinations of alkanes, fatty acids, primary alcohols, aldehydes, ß-diketones and secondary alcohols (Jetter et al., 2006; Bargel et al., 2006; Reiderer and Markstadter, 1996). The chemical composition of the plant wax is extremely variable, and these differences can been seen through different species, the organs of species (e.g. leaves to stems) and during organ development (Jetter and Schaffer, 2001).

The compositional analysis of the cuticle is usually described in percentages of chemical compounds or chemical groups of the compounds previously mentioned. Very few species have been analyzed, and because of this, there are possible gaps in the understanding of the plant cuticle. Depending on species, very broad or very specific chemical groups are found in the cuticle. For example, soybean (*Glycine max* L. Merr.) was shown to have a broad range of alkanes, primary alcohols, triterpenoids, and unknowns with no one group being extremely more than others (Kim et al., 2007a). In contrast, Cameron et al. (2006) revealed tobacco (*Nicotiana tabacum*) to contain a C_{31} alkane comprised 75% of the total wax load of fully expanded leaves. It was reviewed that high percentages of either primary alcohols or ß-diketones have been found in many Poaceae species (Jetter et al., 2006).

Morphology of the Plant Cuticle

The outer most waxes of the cuticle layer are termed epicuticular waxes. These waxes have been studied extensively since the introduction of the SEM in the 1960s, for their diverse morphology and relation to the protection of the plant. Many of the functions that the cuticle gives to the plant are directly related to the morphology of the epicuticular wax layer, especially the wettability of the plant surface.

There have been two extensive reviews discussing the topography and morphology of epicuticular waxes and the variability that can be seen from species to species (Barthlott et al., 1998; Jeffree, 2006). Through these reviews the authors identify many of the forms that epicuticular waxes develop, and discuss the differences among them. Barthlott et al. (1998) (Fig. 2.2.) breaks the epicuticular wax forms into seven categories: films, layers and crusts, granules, platelets and plates, rodlets, threads, and tubules. Most of these categories are broken up further in to sub groups depending on the variations within each category. Jeffree (2006) gives an excellent review of these categories, as well as, numerous plant species that each morphological type of epicuticular wax form can be found.

These epicuticular wax forms are mostly studied using scanning electron microscopy (SEM). Most of the waxes form 3-dimensional structures on the surface of the plant, these structures have been termed, crystalloids. Crystalloids, typically, are characteristic of certain taxa and their shape is reasonable constant (Barthlott et al., 1998). The morphological shape that some waxes take has a direct relationship with the chemical composition of the wax. An example involves *beta*-diketone tubules that can be found on many Poaceae species. This common tubule consist mainly of hentriacontan-14, 16-dione, which are *beta*-diketones, usually found between 2 and 3 micrometers long and 0.2-0.3 micrometers in diameter (Jeffree et al., 1975; Barthlott et al., 1998; Bargel et al., 2006). Plates and plate-like structures are one of the most common categories in the plant kingdom. These structures can be found in *Eucalyptus*, many grasses, and *Fabaceae*. Although, the structures are under the category of plates and are very similar in shape, the plate morphologies can be modified by certain chemicals found in the cuticle, such as, alkanes, aldehydes, esters, ketones secondary alcohols and fatty acids (Jeffree, 2006).

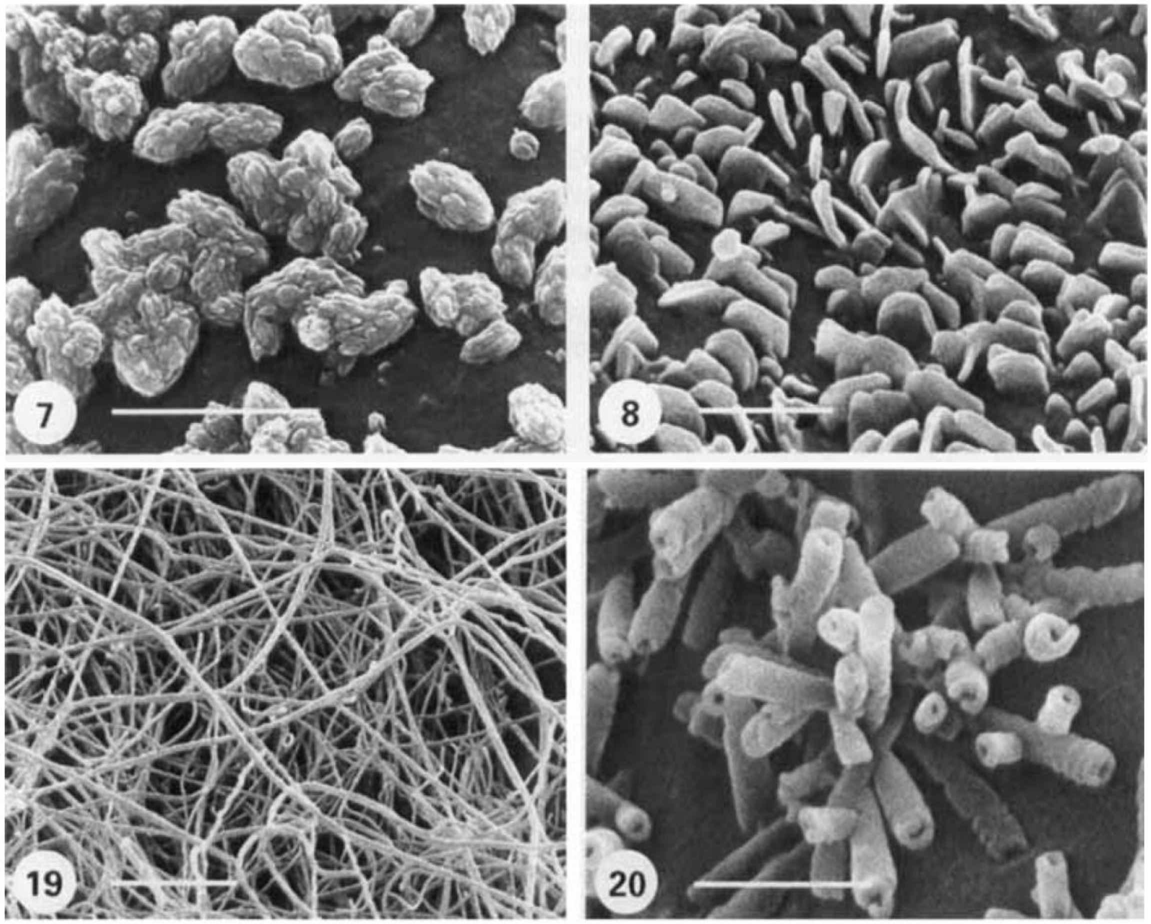

Figure 2.2: Scanning electron micrographs showing various plant cuticle crystalloid morphologies. 7: Granules: *Aegiceras corniculatum* (L.) Blanco, Scale bar =5µm. 8: Entire platelets: *Habropetalum dawei* (Hutch. & Dalziell) Airy Shaw, Scale bar = 1µm. 19: Threads: *Drosera burmanni* Vahl, Scale Bar = 2µm. 20: Tubules: *Lonicera korolkoxii*, Stapf, Scale bar = 1µm. (Barthlott et al., 1998).

The epicuticular waxes of the cuticle layer also have the ability to self-assemble. Self-assembly is the process of structuring in biological systems, where particles or other units interact and self organize to form well defined structures (Benyus, 1997). Studies have revealed that when cuticular waxes are removed and dissolved in organic solvents, and then allowed to recrystallize, the structures formed are similar in shape to the ones seen on the plant surface (Jetter and Riederer, 1994; Neinhuis et al., 2001; Koch et al., 2006). As noted above, the structure that is formed or reformed is directly related to the chemical composition of the wax.

Wettability of the cuticular layer is important aspect of the leaf surface and plant life. Contact angle, of a water droplet and the leaf surface is a measurement of wettability. It is measured as the angle of the tangent plane of a water droplet and the leaf surface at the contact point of the three phase boundary where liquid, solid and gas intersect (Sherpherd and Griffiths, 2006). Leaf surface contact angles below 90^o are considered wettable (hydrophilic), while contact angles above 90^o are usually called non-wettable (hydrophobic) (Bargel et al., 2006). Contact angles can be related to water retention capacity, which is significant for foliar deposition and uptake of nutrients (Cape, 1996). With the study of cuticles by scanning electron microscopy, it was shown that plant micro- and nanostructures, such as, cellular protrusions, cuticular folding and epicuticular waxes reduce the contact area between water and the plant surface (Barel et al., 2006).

Environmental Influences on the Plant Cuticle

Plants are stationary organisms; they are unable to change their location to protect themselves or to ensure their survival. Because of this problem, plants have evolved and developed countless ways of dealing with their environment so they can live and reproduce. One of the ways that plants adapt to their environment is changing the cuticle layer to serve the plant as best as possible. There have been numerous studies and literature on the way the cuticle develops and adapts throughout the plant's life cycle and adjusts to various environmental conditions. This section will discuss some of the adaptations the cuticle goes through.

The most investigated environmental influence on the cuticle has been drought stress or water deficiency. There have been numerous studies conducted on various plant species looking at the change in cuticle wax load due to drought stress. The effect of drought stress and periodic drying events has been well documented on various plant species (Bondada, et al., 1996; Kosma, et al., 2009; Cameron et al., 2006; Kim et al., 2007a, 2007b; Jenks et al., 2001; Seiler, 1985; Kosma and Jenks, 2007; Samdur et al., 2003). Similar results were found in these studies showing an increase in cuticle wax accumulation due to reduced water stress. This indicates the plant response to drought stress is an increase in cuticle wax to reduce cuticular transpiration and thus reduce water loss. Also, drought resistance plants, such as those adapted to arid conditions usually have a thicker cuticle layer compared to plants that live in more temperate climates (Shepard and Griffiths, 2006).

Another stress that causes similar responses like drought in the cuticle is salinity. The way salinity stress is similar to drought, is that ion accumulation in the soil of sodium, chloride and others reduces the water potential making it difficult for the plant to absorb water from the soil. An increase in wax deposition to the cuticle has been shown due to salinity treatment in plants. In *Arabidopsis* plants treated with 150 mM NaCl an 80% increase in total wax accumulation was seen (Kosma et al., 2009). Jordan et al. (2001) suggested that a pattern of increasing wax to the cuticle on ornamental trees was due to irrigation of effluent (reuse) water. The large cuticles found in many plant species adapted to saline conditions and salt marshes not only help with reduce transpiration decreasing water loss and ion uptake, but it is suggested by authors that the cuticle is adapted to repel saline water droplets from the plant surfaces, and reduce salt ion uptake through leaves (Kosma and Jenks, 2007).

Because of culturing plants in the greenhouse, the effect of humidity has been studied to test survival of plants once removed from the greenhouse setting. Shephard and Griffiths (2006) reviewed that high humidity and low light intensity for tissue culture suppresses wax production on plants making them susceptible to desiccation. Koch et al. (2006) studied three plants species with different surface wax compositions and found a pattern that relative humidity (RH) influenced wax accumulation. The studied also revealed that a change in crystalloid density was affected by RH treatment.

With turfgrasses constantly needed regular maintenance such as mowing, rolling, and grooming, mechanical stress is an ever present factor influencing the cuticle layer. Wax can be removed by windy conditions, impact of raindrops, dust, snow, leaf to leaf contact (Shepherd and Griffiths, 2006). Broccoli (*Brassica oleracea* var. botrytis) was tested for wind abrasion and brushing effects on conditioning of the cuticle. After 9 d treatment a similar reduction of 31-38% was seen for brushing and wind abrasion. But 15d of treatment the reduction went to 15% and 6% for brushing and wind, respectively. This indicates wax recovery from the abrasive conditioning (Latimer and Severson, 1997). The regular grooming practices that turfgrasses undergo, could have a significant impact on the morphology and total wax load of the cuticle.

Cuticle Regeneration

Although, waxes can be removed from the leaf surface through various means, as previously mentioned the plant has the ability to reproduce and recover the cuticle extremely quickly. Recent technological advances and research have given some light into the subject of wax regeneration. Koch et al. (2009) using atomic force microscopy (AFM) showed the importance of the cuticle to the plant as it starts to regenerate immediately after it was removed for every species they studied. Also, waxes grow in layers and start to produce crystalloids quickly within this process. It was shown that wax regeneration speed and amount differ. *Galanthus nivalis* regenerated a multi-layered wax film, only 80 min after the removal of wax, but for *Euphorbia lathyris* it took 20h before a multi-layered film could be detected (Koch et al., 2004). This could prove important to turfgrasses with the mechanical stress and removal of waxes from maintenance.

Foliar Fertilization

Foliar fertilization is a staple in many turf managers' maintenance programs that is greatly utilized throughout each year. Applications of foliar nutrient solutions have been conducted and studied over the last century. Recently with the development of improved fertilizer formulations, enhanced application equipment and increasing environmental concerns foliar fertilization has become more important than ever. These foliar applications allow for a more accurate application, a quick plant response, and lower input level. Although, this practice is very popular there are still many unanswered questions about the mechanisms involved with the penetration of the fertilizer into the plant.

In the beginning, field studies with foliar sprays were conducted mainly with fruit investigating various nutrient sprays. Since then the effectiveness of foliar applications have been accessed on many different crop species, determining penetration and availability rate, phytotoxicity, correction of deficiencies, physiological processes and effect of quality and yield response (Fernandez and Eichert, 2009). Research has mostly utilized agricultural crops where the main parameter of measurement has been yields. With turfgrasses other parameters must be used to assess the benefits of foliar applications, and there have been a number of studies evaluating these benefits.

One of the benefits of using foliar fertilizers, especially for lower mowing heights, include a total fertilizer input that is lower than if you would use a 100% granular fertilizer program (Liu et al., 2005; Totten, 2006). For creeping bentgrass, fungicides are applied very often, and foliar fertilizers provide an excellent way to tank mix and apply two treatments with the same application. Nutrient deficiencies can rapidly be corrected for macro- and micronutrients when using foliar fertilization (Marschner, 1995). When the turf is under stress, low rates of foliar fertilization can be applied without increasing plant stress or foliar burn. When soils have a low CEC, like sand based putting green root zones, foliar fertilizers are beneficial because the soil is unable to hold as many nutrients. Any soil condition where root growth or acquisition of nutrients is hindered, foliar fertilizing is a good way to provide the plant with nutrients. Situations such as, improper irrigation or water depth, salinity stress, soil pH and compaction, air and soil temperatures and mowing height can have an effect on root growth and development, leading to decreased nutrient uptake.

Applying turf with low rates of nutrients on a regular basis promotes uniform growth that increases playability and aesthetics (Bowman, 2003). Schlossberg and Schmidt (2007) revealed that frequently fertilizer applications using a pressurized sprayer increased canopy color, leaf nitrogen concentration, and leaf micronutrient concentrations of a putting green with creeping bentgrass and annual bluegrass(*Poa annua*). A recent study on ammonia volatilization of putting greens applied with foliar applications, revealed very low losses, giving evidence of reducing losses of fertilizers to the environment by foliar feeding (Stiegler et al., 2011). McCarty (2011) states that foliar feeding can minimize growth surges, enhance ball roll, minimize thatch/mat accumulation, and maintain turf color during the summer months on sand based putting greens.

Factors Influencing Foliar Absorption

There are many factors that are involved in the penetration of foliar applied solutions. Leaf surface chemical and physical properties, environmental conditions, solution concentration, adjuvant addition, as well as others, all have a direct affect on the mechanism of foliar uptake. This has been part of the problem when studying foliar absorption because the variation in the results all can be traced to the inconsistent experimental conditions that were used.

The wettability is closely related to surface morphology. By studying the effects of surface roughness on wettability, Yoshimitsu et al. (2002) (Fig. 2.3.) found that as surface roughness increased the contact angle of a water droplet increased on small manufactured pillars. Since the development of the scanning electron microscopy scientist have been able to study plant surfaces, and found that the epicuticular wax structures play a major role increasing the contact angle of water on the plant surface (Bargel et al., 2006). Koch and Ensikat (2008) reviewed that retention rate and wettability was determined by epicuticular wax occurrence and leaf surface characteristics. It has been shown that by changing humidity you can affect crystalloid size and density which resulted in a change in contact angle (Koch et al., 2006). This increase in epicuticular structures could lead to reduced adhesion of a water droplet of a foliar applied solution. Leaf topography, shape and position of the plant leaf all have shown to have an effect on surface wetability of a water droplet (Magarey et al., 2005).

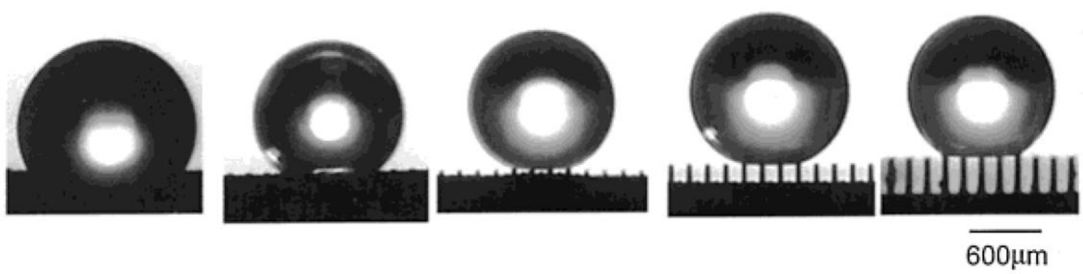

Figure 2.3: Diagram demonstrating surface roughness affects water droplet contact angle. As roughness increases so does contact angle. (Yoshimitsu et al., 2002)

As with most plant experiments and practices the environmental conditions play a significant role in foliar uptake of a solution. There are direct affects on the absorption of the solution as it is being applied, like temperature, RH, and irradiance. They all play a significant role in how the spray interacts with the leaf surface and the uptake and allocation of the solution (Fernandez and Eichert, 2009). Relative humidity has been shown to have a major impact on the absorption of foliar applied solutions. As humidity increases, the amount of ions or salts in the foliar applied solution entering the plant increases as well (Ramesy et al., 2005). On grapefruit (*Citrus paradise* Macfad.) leaves, urea penetration was increased by 50% as the RH increased from 20-35% to 35-50% (Orbovic et al., 2001). Temperature can affect foliar absorption by increasing metabolic activity inside the plant (Marschner, 1995). Orbovic et al. (2001) also found that as temperature increased from 19^{o}C to 28^{o}C the penetration of urea was higher, but there was no difference between 28-38^{o}C. One explanation the authors presented was the droplet drying time decreased from the 25min to 11min for the 19^{o}C and 38^{o}C treatments, respectively.

In contrast, there have been studies that reveal temperature did not affect foliar absorption. In a two year study, Stiegler et al. (2010) found there was no correlation between air temperature and foliar absorption of urea on a creeping bentgrass green. There was no temperature effect in the range of 10^{o}C to 25^{o}C for penetration of potassium through astomatous cuticles of pear (*Pyrus pyrifolia*) and citrus leaves (Schonherr and Luber, 2001). The hydrophilic compound xylose showed no differences in the penetration through ivy (*Hedera helix)* leaf cuticles due to temperature changes (Popp et al., 2005). This demonstrates some of the discrepancies in the literature to changes in the environment affecting foliar penetration.

Stomatal Pathway

It has long since been believed that stomata play a significant role in the penetration of foliar applied solutes into the plant. With improvements in technology, it is possible to investigate more accurately the impact stomata play in foliar absorption. A study investigating humidity, light, stomatal density and re-wetting on uptake of ionic solutes showed increased uptake with humidity, stomatal aperture and density (Eichert and Burkhardt, 2001). A recent publication, suggests that nitrogen uptake is influenced by stomatous leaves and stomatal aperture with results showing an increase of nitrogen uptake due to increased stomatal aperture (Eichert and Goldbach, 2008). These results indicate that the stomatal pathway is a major way for uptake of foliar applied solutes.

Although there is evidence to support stomatal uptake of solutes, there are other data that support cuticular pores providing pathways as well. Studies with inorganic ions and various plant species revealed that penetration through astomatous cuticles does and can occur, which gives evidence to cuticular polar pores involved in foliar uptake (Schonherr, 2001; 2002). Eichert and Goldbach (2008) observed that although there was more penetration of urea on through stomatous leaves, there was still uptake through astomatous leaves, as well. The results in these studies provide us with evidence of a cuticular pathway for foliar uptake, and that more research is needed to continue our understanding of the cuticle as a barrier to foliar applied solutions.

Surfactants

It is very common for chemicals to be added to the solution of foliar applications to increase the efficiency. Surfactants and adjuvants have been used for many years, over many different crops and plant species. Surfactants are used to increase the uptake of a chemical or fertilizer by increasing the droplet spread of the solution on the intended surface. Much of the research has studied the effects of surfactants increasing herbicide retention in plants with little investigation with fertilizer solutions. The results suggest surfactants increase herbicide retention and should do the same for fertilizer uptake.

A study investigating primisulfuron on barnyard grass [*Echinochloa crus-galli* (L.) Beauv.] and green foxtail [*Setaria viridis* (L.) Beauv.] observed an increase in spread of a droplet applied to the leaf surface with a non-ionic surfactant added, compared to the droplet without a surfactant. They also observed even greater spread when an organosilicone wetting agent was added to the solution too (Sanyal et al., 2006). Liu (2004) revealed that adding surfactants to the solution and an increase in glyphosate and 2-4D was seen in various plant species, but that the mechanisms are more complex than previously believed. Field research studying the foliar uptake of potassium in cotton suggested that surfactants may enhance K uptake compared to K applied in a water alone solution (Howard and Gwathmey, 1995). Control of itchgrass (*Rottboellia cochinchinensis* L.) was observed in corn with the use of various surfactants and adjuvants, with the results depending on the combination of herbicide used and adjuvant added (Strahan et al., 2000).

Although there is research that suggests surfactants work well, the results have been inconsistent. Reickenburg and Pritss (1996) studied urea uptake in red raspberry (*Rubus idaeus* L.) and revealed the addition of a surfactant did not increase uptake levels of the foliar applied solution. Control of crabgrass [*Digitaria ischaemum* Schreb.] in cool-season turfgrass was studied and found that adding a surfactant did not increase control under normal conditions, but the authors mention improved efficacy was noticed with surfactant on a droughty site (Neal et al., 1990). These results suggest that the surfactant could help the herbicide retention on the plant that is experiencing stress unable to absorb the herbicide because of changes due to the stress. Research has shown that surfactants can be variable in their efficiency, as mention earlier Liu (2004) revealed the combination of surfactants and the intended chemical was a complex interaction. The structure and chemistry of the added surfactant play a significant role in how the surfactant will increase the uptake of the solution and should be investigated before choosing which adjuvant to use with your solution (Wang and Liu, 2006).

Transgenic Plants

Transgenic plants are plants that have been genetically engineered, and techniques of recombinant DNA are used to create plants with new characteristics. This technology has been useful in laboratories to study certain processes and mechanisms in plants and to increase our knowledge about the plant. This allows researchers to learn a great deal about the plant's mechanisms and biological processes. Over the last two decades transgenic plants are now being used in agriculture to increase yield and stress tolerance. There is much debate on whether these genetically modified plants should be used in a natural, commercial setting, although there are many opposing the sale of these plants, research with transgenic plants is now larger than ever (Snow and Palma, 1997).

The future of the turfgrass industry could be ideal for transgenic research, as the commercial industry is always looking for a grass with better color, stress tolerance, pest tolerance and low maintenance. A turf that could tolerate extreme salinity stress on the coast, or a bentgrass that has heat tolerance are examples of plants that could provide managers with ways of lowering cost and increasing playability and aesthetics. Guo et al.

(2003) produced a transgenic creeping bentgrass that showed delayed dollar spot (*Sclerotinia* homoeocarpa) symptoms in field trials compared the control plants. By using barley *hva*1 gene, a transgenic creeping bentgrass was produced that showed signs of drought tolerance (Fu et al., 2007). Xu et al., (2009) produced transgenic creeping bentgrass that had significantly enhanced heat tolerance compared to the control lines, which is a very important and interesting trait for creeping bentgrass in the transition zone. The advantages of transgenic breeding are breeders can target exactly the trait of interest they want to introduce to the plant and it is a much faster process than traditional breeding. There are drawbacks though, as morphological changes have been seen when transgenic plants are produced, and the threat of the genetically modified DNA breeding with other plant species in the natural setting (Reichman et al., 2006). Because of the last drawback increased research is needed to understand how these modified plants react and change under various environmental conditions.

Currently, researchers at Clemson University are studying transgenic creeping bentgrass and producing plants with increased drought and salinity tolerance, as well as pest tolerance. Li et al. (2010) recently published an article describing a transgenic creeping bentgrass expressing an *Arabidopsis* vacuolar H^+-pyrophosphatase (AVP1) gene, which increased salinity tolerance in the turfgrass. A recent field study with the same transgenic grasses was just concluded with interesting results with the transgenic salinity tolerance in field settings. Although this grass did show significant salinity tolerance, there were morphological changes to the turf compared to the wild-type (WT). This could mean other processes or parts of the plant were changed as well. For this reason we must study and research genetically modified plants as much as possible before commercializing them, and allowing them into the industry.

Fractal Dimension of Hydrophobic Surfaces

Since Benoit Mandelbrot coined the term fractal in the 1970s the use of fractal analysis and dimensions has been increasingly popular. Fractal analysis can be used in a wide range of research and sciences from botany to finances and everything in between. Mandelbrot came up with *fractal* from the Latin adjective *fractus* and the corresponding Latin verb *frangere* which means “to break”, or to create irregular fragments (Mandelbrot, 1977). The name also refers to complex shapes that can be described as the fractal dimension, which is normally a fraction.

To understand fractals or fractal analysis it is necessary to have some basic knowledge and background of fractals. Fractals can be defined as objects of self-similarity or structures that are self-similar, scale-invariant (Masters, 2004). The term self-similar simply means that as you look at an object at different magnifications or scales, the objects appear to look the same. Koch snowflake and Sherpkiskis triangle are examples of fractals that are the very definition of self-similar (Fig. 2.4.). Nature provides us with many interesting fractals, they are not perfect as in the examples above, but there are self-similarities that can be called fractals. Two examples that come up often in the literature are trees and their branches and coastlines. For example, if you use a coastline on a map, it may look slightly convoluted, but then magnify an area on that coastline and it will look convoluted much like the beginning picture. You can repeat this theoretically, all the way down to the atoms of the coastline and you will continue to get a convoluted rough line that looks much like the beginning coast line on a map. Trees also have this same property of self-similarity. If you can image a tree with no leaves on it, the branches grow out from the truck. Then look closer at a branch and smaller branches grow out from it, which looks similar to the trunk and first branches. You can continue this process to the smallest branches and the self-similar property will continue.

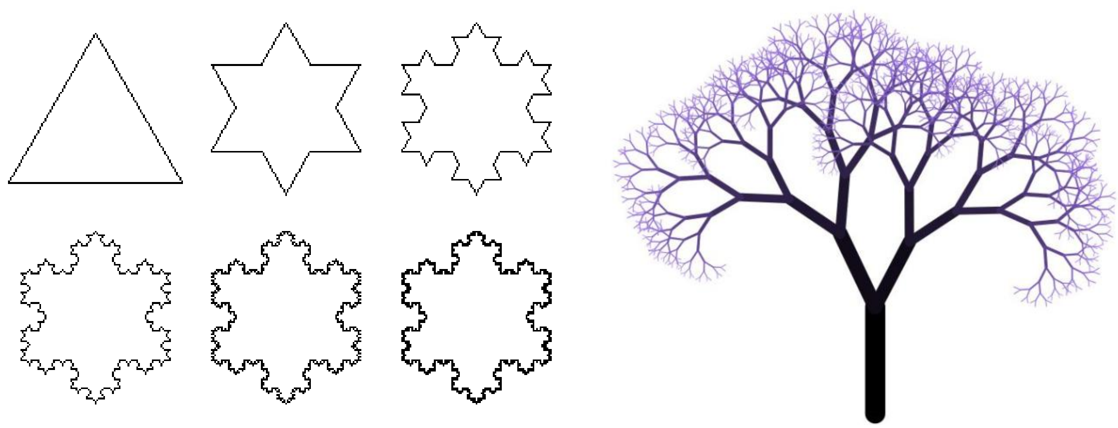

Figure 2.4. Example Koch's Snowflake after five iterations, and a diagram of a tree showing the fractal nature and self-similarity of objects.

Now only objects like Koch's snowflake (above) are examples of exact fractals, where self-similarity applies over an infinite scale range. As mentioned, fractals are found everywhere in nature, coastlines, mountains, leaves, and other rough surfaces, but these are not perfect fractals like the equations described by Koch and Sierpinsky. These natural fractals are not perfect fractals and are considered statistically self-similar (He et al., 2007). These objects that have been described are irregular and convoluted and Euclidean geometry does not apply to these objects because their measurements are scale dependent. Because of this, fractals cannot be described with an integer and a non-integer dimension must be used which is called the fractal dimension (Feder, 1988). Not only is this characteristic of self-similarity interesting to think about, it can be mathematically analyzed and provide valuable information using the fractal dimension. The analysis that can be utilized to study fractals and fractal structures is called fractal analysis. One way the fractal dimension can be utilized is measuring and classifying hydrophobic and super hydrophobic surfaces related to surface roughness, which will be discussed further in this chapter.

To understand what it means to be scale-dependent, one must think about how the measurement depends on the resolution. Bassingthwaighte et al. (1994) stated “The valued measured depends on the measurement resolution. There is no one ‘true’ value for a measurement” in their book Fractal Physiology. The authors were describing how the measurement of an object depends directly to the measurement resolution. For instance, measure a coastline and use a measurement tool of 1km. Then take that exact same coastline and use a measurement tool of 10m, the coastline measurement will not only be more accurate but it will be increased in length. The same will happen by decreasing the tool to 1m, the coastline will continue to get longer and also provide a more accurate measurement. So by measuring a fractal with a finer unit of measure, the contours and irregular shape can be better estimated, with increased accuracy (Sztojanov et al., 2009). This progression of measurement resolution is called the scaling relationship (Bassingthwighte et al., 1994).

A popular method of determining the fractal dimension of objects closely related to measurement resolution is called the box-counting method. Box-counting is a procedure used to determine the fractal dimension of a particular object or fractal. An image is used and a grid with a precise box size (*s*) is laid over the image. Then 'count' the number of boxes (*N*) that has a piece of the object of interest. Repeat this with more boxes with smaller size. A log-log plot of the number of boxes counted by box size for each step is plotted, and then a line is fit through the plots. The resulting slope of the line is known as the box counting dimension that corresponds to the fractal dimension- D*f* (He et al., 2007; Masters, 2004; Sztojanov et al., 2009; Feder and Aharony, 1989). The equation is as follows:

$$Df = \frac{\log N(s)}{\log (1/s)}$$

D*f* = the fractal dimension (slope of the line)
N = Number of boxes counted with object of interest
s = size of box for that step

As mentioned earlier, researchers can use the fractal dimension for many different measurements, from classification to describing morphology. An example is the classifying of plants in the Gentianaceae family using transverse section images of the root, stem, and leaf of different species (Sztojanov et al., 2009). Another example is scientists are using fractal analysis to classify lesions found in women's mammograms. As the object becomes more irregular the fractal dimension increases. Using this information they determined a fractal dimension of lesions below 1.4 were mostly benign, while lesions with a fractal dimension above 1.4, 92% of the cases were malign (Sztojanov et al., 2009). Discussing the branching of the vascular tree in the human retina, the author proposed models for optimal formation and pattern of the human bronchial tree, with possibilities of developing bioartificial organs (Masters, 2004). Kaye (1989) has a chapter that discusses different uses for fractal analysis, from the mining industry, cosmic fine particles, and types of sand and respirable dust.

A common way to use fractal analysis and technique that is utilized in the current thesis is studying surface roughness in relation to hydrophobicity. There is a great deal of interest in materials science as how surface roughness creates a hydrophobic surface that can repel water very well. This has been studied in plants and is called the "Lotus Effect" from the plant it was discovered on and it continues to get much interest from other fields of research. Research on ploy (alklpyrrole) films revealed that as the fractal dimension increased from 2.03 to 2.18, the contact angle of a water droplet increased from 125^{o} to 154^{o} (Kurogi et al., 2007). Onda et al. (1996) used alkylketene dimer (AKD) to demonstrate a fractal surface can produce superhydrophoic surfaces. The authors estimated the fractal dimension to be 2.29 which when measured had a very high contact angle as large as 174^{o}, super hydrophobic. Ramos et al. (2009) investigated the relationship between surface roughness and fractal dimension. They found that as the surface roughness increased 1.14-3.10 the fractal dimension increased from 2.18-2.69. The authors also revealed that each of the fractal surfaces produced contact angles that cause the surface to be considered hydrophobic.

Current research in various fields is now looking at this relationship of surface roughness and how it relates to the hydrophobicity of materials. Feng et al. (2002) provides a brief review of how the knowledge of this relationship is being utilized from studying plant leaves and applying this knowledge to textiles, coatings, gene delivery, and non-wetting transfer. Silicon oxide nanowires were produced and investigated for their super hydrophobicity, and it was determined that the hydrophobicity (contact angle $> 150^o$) was a result of the porous nature and high surface roughness that had developed (Coffinier et al., 2007). From the literature provided above, one can see that rough, irregular surfaces that are fractal in nature provide hydrophobic surfaces which in turn decrease the wettability of these surfaces. This information can be useful when studying the morphology of the plant cuticle and possibly giving insight into how the crystalloids protect the plant.

CHAPTER III

CUTICULAR RESPONSE OF CREEPING BENTGRASS TO DROUGHT AND ITS INFLUENCE ON FOLIAR APPLIED ^{15}N UREA

Introduction

Drought stress is a common occurrence on many crops and plant species of un-irrigated agricultural areas. Golf course superintendents and turf managers for the most part have the luxury of an irrigation system in place to provide the plant with water to match evapotranspiration (ET) rates. With increasing drought conditions as Dai (2011) predicts, irrigation restrictions will increase and limit the amount of water available for use on turfgrass. A popular management technique used especially on golf greens is reducing the water for a period of time to manage the playability of the surface. This can cause moderate drought stress symptoms to the plant, modifying the morphological and physiological processes in the plant. Turf managers must learn how to manage the plant under stressful conditions, and understand the changes developed during drought conditions to enhance the management programs.

Foliar fertilization has been developed as an excellent way of providing quick response and nutrient additions to the turf in an accurate and reduced labor manner. Research has shown that at combining liquid and granular provides the best results to creeping bentgrass, instead of exclusive use of one method (Totten et al., 2009). It has also become common place to apply fertilizer in tank mixes with other foliar chemicals, such as PGR’s, and pesticides. A number of programs and strategies for various chemicals have been developed and to stay up to date with the timing of applications, managers may have to apply during stressful conditions.

The plant has evolved many different morphological and physiological processes to defend itself from stressful conditions. Leaf area and orientation are two responses that occur when the plant senses reduced water. Various plants also have the ability to adjust its osmotic potential to reduce its overall water potential and up take water under drought conditions. Another important way the plant adjusts during drought stress is the cuticle layer. It has been shown over numerous studies that the cuticle increases due to drought or periodic drying events (Bondada et al., 1996; Kosma et al., 2009; Cameron et al., 2006; Kim et al., 2007a; 2007b; Jenks et al, 2001; Seiler, 1985; Kosma and Jenks, 2007; Samdur et al., 2003).

The plant cuticle acts as a barrier to excessive water loss from inside the plant, but also hinders pollutants and other chemicals from entering the plant. The morphology of the cuticle plays a major role in various defensive mechanisms to protect the plant. Bargel et al. (2006) reviewed the cuticle provides: protects from uncontrolled leaching of ions, water repellency, anti-adhesive, host-pathogen signaling, reflection of UV-radiation and mechanical resistance. The authors also suggest because of its morphology and chemical composition, the cuticle provides an excellent barrier to foliar applied chemicals and hinders foliar uptake.

The objectives of this study were to 1) investigate the changes in composition and morphology to the cuticle layer of creeping bentgrass under two drought levels; 2) determine if the effects seen from drought stress on the cuticle negatively influence the absorption of foliar applied ^{15}N-labeled urea.

Materials and Methods

'A1-A4' creeping bentgrass 15.24cm plugs were sampled December 15th, 2010 from Thornblade Country Club's nursery putting green which was established on 85:15 (sand:peat) USGA specification rootzone mix. The plugs sampled were planted in black plastic pots with 85:15 rootzone mix and placed in a growth room at Clemson University Greenhouse Facility, Clemson SC. The plugs were given approximately 1 month to establish and acclimate to the growth room. The samples were given a 14-14 P_2O_5-14K_2O granular fertilizer at a rate of 4.8N g m^{-2} at planting, and then on a bi-weekly basis the turf was fertilized foliarly at 0.98N g m^{-2} with Progressive Turf's 10-3-5 liquid fertilizer. The plants were irrigated once daily for a week during establishment then reduced to 3-4 times a week. The pots were also mowed at least 3 times a week with electric clippers at approximately half an inch clipping s removed. The average temperature during the acclimation and experimental period was 22.7^{o}C with a max and min of 33 and 20^{o}C, respectively. During that same period the relative humidity averaged 56% with a max and min at 76 and 22%, respectively.

Irrigation Treatments

The irrigation treatments consisted of a control, where 100% of the average evapotranspiration rate (ET) was replaced daily, and two drought levels, level I and level II where 50% and 25% ET was replaced daily, respectively. ET rates were based off average daily ET rates and determined gravimetrically over a 4 day period. Experimental units were irrigated to saturation, 1h later the weight was taken for individual pots, and again at 24h, 48h, and 72h. Average weight loss of each day was determined to be daily ET rate. To prevent run off of treatments, the irrigation treatments were applied with a 100ml syringe. Once treatments commenced they lasted for 10d and on the eleventh day sampling took place.

Cuticle Composition and Quantification

Cuticle analysis methods based off Jenks et al., (1995) with modifications. One gram subsamples of fresh creeping bentgrass clippings (2 separate samples per experimental unit), were submerged in approximately 25ml of hexane for 50s to remove the cuticle layer. The extract was poured off into a 25ml glass tube for further preparation. Extracts were evaporated to dryness under a nitrogen stream. Sample was dissolved in approximately 1ml of hexane and transferred to a concial vial. Extracts were evaporated to dryness once again. Residues were derivatized by heating at 100^{o}C for 20min using bis(trimethylsilyl)-acetimide (BSTFA:Sigma-Aldrich). Silyated samples were analyzed by gas chromatography (GC) with an Agilent Technologies GC 7890A equipped with an Agilent Technologies5975C Mass Spectrometer (MS). The GC has a DB5 MS 30m × 250µm × 0.25µm film column with helium as a carrier gas. The initial temperature was 50^{o}C held for 2min, then increased 40^{o}C min^{-1} to 200^{o}C, at which point it began to increase 3^{o}C min^{-1} to 300^{o}C, where it remained unchanged for 6 min. 2µl of sample was injected, with the injector temperature set at 250^{o}C and a constant flow of 1µm min^{-1}. Quantification and analysis was based on Electron Impact Mass Detector (EIMD) scanning from 40-500 atomic mass units (amu), with the detector being turned on at 5min. All quantification of cuticle chemicals were based on comparison of area to the internal standard/surrogate tetracosane (10μg) added during sample extraction. Three common standards commonly found in the plant cuticle were also ran, an alkane (C_{21}-C_{40}: Simga Aldrich), a primary alcohol (C_{26}: Sigma-Aldrich) and fatty acid (C_{26}: Sigma-Aldrich). For peak identification, mass spectra from peaks in samples were compared to the standards and spectra in the Wiley NIST library.

Surface Area Calculation

Creeping bentgrass leaf surface area for wax extracts was calculated using WinRhizo software (Regent Instruments, Quebec). Fresh clippings were harvested, weighed and immediately placed on the machine for analysis to avoid curling of leaf blades and an incorrect measurement. This was repeated at least six times for each treatment. This measurement developed an average surface area we used for the 1g of fresh weight for the cuticle extraction.

Scanning Electron Microscopy

Micrograph images of creeping bentgrass cuticle layer morphology were acquired using a Hitachi SU6600 Field Emission Scanning Electron Microscope (FESEM) at The Clemson EM Facility in Pendleton, SC. Samples were taken and fixed on aluminum stubs with carbon tape with adaxial side up, and allowed to air dry for 12-24h before imaging based on methodology from (Pathan et al., 2008). Samples were sputter coated with platinum and then analysis and images were acquired. Microscope was set at 5kV and a working distance of 10mm. Images were acquired at 2.5, 5, 10, 20, and 30k magnification to study cuticle morphology and crystalloid shape.

Crystalloid Density

Micrograph images of creeping bentgrass cuticle morphology were examined for differences in the density of crystalloids covering the cuticle layer based on methodology by Beattie and Marcell (2002). Images were acquired at 10,000x magnification. Images were adjusted to a uniform brightness and contrast before analysis. Then images were analyzed using NIS Elements (Nikon) by thresholding and object count to determine the % area the crystalloids covered by treatment.

^{15}N Foliar Absorption

^{15}N-labeled urea (ICON Stable Isotopes, Marion, NY) at 2.79% atom enrichment of ^{15}N was used for the study. The ^{15}N-labeled urea was applied 10d after irrigation treatments. The fertilizer was applied before 800h using a spray chamber to ensure uniform delivery of the solution. The rate of ^{15}N-labeled fertilizer was 0.732N g m^{-2} with a delivery volume of 561L ha^{-1}. Sampling for tissue analysis was done 24h after fertilization. Sampling was done by splitting experimental units in half, and then one half plant tissue above the thatch layer was harvested, placed in paper bags and allowed time to dry before further analysis took place. The other half of the experimental unit was left for tissue to use for cuticle extraction and analysis. Once the samples were collected then the thatch layer and roots were separated from each experimental unit and allowed to dry before further analysis. Analysis for isotopic ^{15}N in samples and fertilizer was determined at the University of Illinois at Urbana-Champaign using the automated Rittenburg technique (Mulvaney et al., 1990), on a Nuclide/MAAS 3-60-RMS double mass spectrometer (Nuclide Corporation, Bellefonte, PA).

Statistical Analysis

The experiment was a completely randomized design. Each treatment had a total of six replications. Data were analyzed using JMP 9.0 statistical software (SAS, Cary, NC). Analysis of variance was used to test treatment effects ($p<0.05$). Student's t test was utilized to separate means further.

Results

Cuticle Composition and Quantification

The effects that drought stress plays on the creeping bentgrass cuticle layer were studied for differences in composition and quantity. Irrigation treatment did not provide any statistical differences in composition or quantity ($p = 0.575$). Although the results were not significant an interesting pattern between irrigation treatment wax totals was as the drought stress level increased, total wax increased as well. Mean wax total for each treatments were 2.77, 2.85, and 3.06 µg cm^{-2} for the 100%, 50% and 25% ET rates, respectively

Along with the quantity of the total cuticle wax, the effects of water stress were studied on wax groups and individual components as well. The cuticle was broke up into 4 different wax groups to get a better understanding of how each chemical reacts to the drought stress, fatty Acids, alkanes, primary alcohols, and a group of compounds that we were unable to identify. The primary alcohol group comprises approximately 92% of the entire cuticle, the other groups or individual compounds comprise 2% or less of the cuticle each.

We found that the three major wax groups (fatty acids $p=0.449$, alkanes $p=0.624$, and primary alcohols $p=0.544$) all reveal the pattern of increasing as the drought stress becomes more severe, but there were no statistical significance. The mean total wax for fatty acids was 0.057, 0.067, and 0.068µg cm^{-2} for the 100%, 50% and 25% ET rates, respectively. The data were similar for the group of alkanes where the means for 100%, 50% and 25% ET rates were 0.0289, 0.0347, and 0.0389µg cm^{-2}, respectively. Primary alcohols (comprising 92% of the cuticle) had total wax means of 2.597, 2.648, and 2.869µg cm^{-2} for irrigation treatments 100%, 50%, and 25%, respectively (Table 3.1.). Many individual constituents present the same type of pattern, but very few have statistical differences.

Cuticle Morphology

The description of creeping bentgrass cuticle morphology and epicuticular crystalloids are based on terminology by Barthlott et al. (1998) and Jeffree (2006). Crystalloids were found covering the leaf surface with the underlying cuticle being relatively smooth. The following description is based on control treatment as a base for cuticle morphology to identify any change due to treatments.

The shape of crystalloids was irregular membranous platelets or primary alcohol plates. Both descriptions are correct based on the publications listed, but because of the cuticle composition being over 92% primary alcohols, the second description is more accurate. Crystalloids were thin, with irregular serrated edges arising from the cuticle. The primary alcohol plates ranged for 1-4µm tall and 1-3µm wide. Crystalloid overall shape did not appear to be affected by drought treatments, but size changed slightly, with crystalloids growing slightly taller and wider in the drought treatments compared to the control (Fig. 3.1).

There was a significant increase in % crystalloid density on creeping bentgrass cuticle due to drought treatment ($p < 0.001$). The % crystalloid density increased significantly for both levels of drought, from 40.9% for the 100% ET treatment to 58.0 and 69.9% for 50% and 25% ET rates, respectively (Fig. 3.2).

^{15}N Foliar Absorption

An average of approximately 40% of the labeled urea fertilizer was recovered with 24% in the leaves and stems, 15% in the thatch layer and <1% in the roots (Fig. 3.3). Irrigation treatments did not influence %^{15}N recovered from clippings ($p = 0.057$) and thatch ($p = 0.619$). Although minimal (<1%) ^{15}N recovered in roots were significantly influenced by irrigation treatments ($p = 0.021$) but no considered biologically significant (Table 3.1)

Irrigation treatments resulted in a significant reduction in total %^{15}N recovery ($p = 0.001$). The means for foliar absorption of ^{15}N were 44.33%, 39.04% and 36.89% for the control (100% ET), drought level I (50% ET) and drought level II (25% ET), respectively (Fig. 3.3.). Correlation of %^{15}N recovery and total wax had a negative relationship ($r = -0.415$), though this relationship was not significant ($p=0.098$).

Discussion

Cuticle Composition and Analysis

The chemical composition of creeping bentgrass cuticle is dominated by the group of primary alcohols, and primarily is made up of 1-hexacosanol. The other 8% of the cuticle is made up of various long chain alkanes, fatty acids, and one aldehyde and a group of unidentified constituents. This is similar to results in barley, where the author found that 89% of the cuticle was comprised of primary alcohols (Giese, 1975). Richardson et al. (2005) found that the principal component in barely leaves to be 1-hexacosanol, which comprised approximately 80% of the cuticle. The results suggest the barley and creeping bentgrass are very similar in composition and possibly react similar to environmental stress.

The drought stress imposed on creeping bentgrass in the present study revealed no significant increase in total wax load, although there was a patterned that wax increases due to water stress. Also, this pattern was not only seen in one compound or one group of compounds, but for the entire cuticle. Gonzalez and Ayerbe, (2010) determined that cuticular wax load of 12 genotypes of barley increased significantly due to water stress treatments, with genotype ND77 increasing 12-20% to the irrigated controls (). Drought and water stress affecting the cuticle has been studied extensively in various plants, and it has been shown that the cuticle wax increases due to drought stress (Bondada et al., 1996; Kosma et al., 2009; Cameron et al., 2006; Kim et al., 2007a; 2007b; Jenks et al., 2001; Seiler, 1985; Kosma and Jenks, 2007; Samdur et al., 2003). The increase in wax is thought to protect the plant from increasing water loss through transpiration.

<u>Cuticle Morphology</u>

Crystalloid density increased significantly in the drought treatments compared to the control. This could be attributed to the pattern of increasing total wax in the drought treatments. The increase in crystalloid density could be a reaction of the plant sensing drought stress and defending itself from excessive water loss. Although this could be protecting the plant from water loss, the increased density could also increase the surface roughness of the cuticle. Surface roughness can affect the contact angle of a water/solution droplet and influence foliar absorption.

Koch et al. (2006) found that low humidity increased the size and density of epicuticular crystalloids on various plant species, which shows the plant reacting to environmental conditions. In the present study we revealed similar results with crystalloids increasing in density due to drought stress. The similar results suggest that perhaps the plant protects itself from water loss by increasing total wax and thereby increasing crystalloid density.

^{15}N Foliar Absorption

The absorption of foliar applied ^{15}N-labeled urea was investigated to determine if changes in the cuticle quantity, composition, and morphology influence uptake. The correlation statistics showed a pattern of increasing total wax results in decreasing foliar absorption, although the correlation was not significant in the present study. One possible explanation is different samples were used for cuticle extraction and ^{15}N absorption from the same experimental unit. Steigler et al. (2010) found a significant negative relationship between foliar ^{15}N absorption and total cuticle wax under field conditions on a creeping bentgrass putting green using similar methodology. Bondada et al. (1997) revealed a highly significant negative relationship between total wax and ^{15}N absorption in cotton when sampling from the same leaves.

Koch et al. (2006) found that as the morphology of the cuticle changed, so did the contact angle of a water droplet, which they attributed to surface roughness changing. Increases in surface roughness like the increase in crystalloid density seen in the present study, could have a negative effect on the absorption of a foliar applied solution, and perhaps increase losses by volatilization/evaporation.

Conclusions

Changes in the cuticle of creeping bentgrass experiencing drought stress can have a significant impact on foliar absorption. The morphology and total amount of the cuticle protect the plant in various ways but present a problem to the uptake of a foliar applied solution. Further research needs to continue to determine the effect of the cuticle on foliar absorption and determine how much morphology and total amount of cuticle wax influence uptake of a solution. Also, research should study if adding an adjuvant, such as a surfactant, will negate the effects drought stress on the cuticle and possibly increase foliar absorption levels.

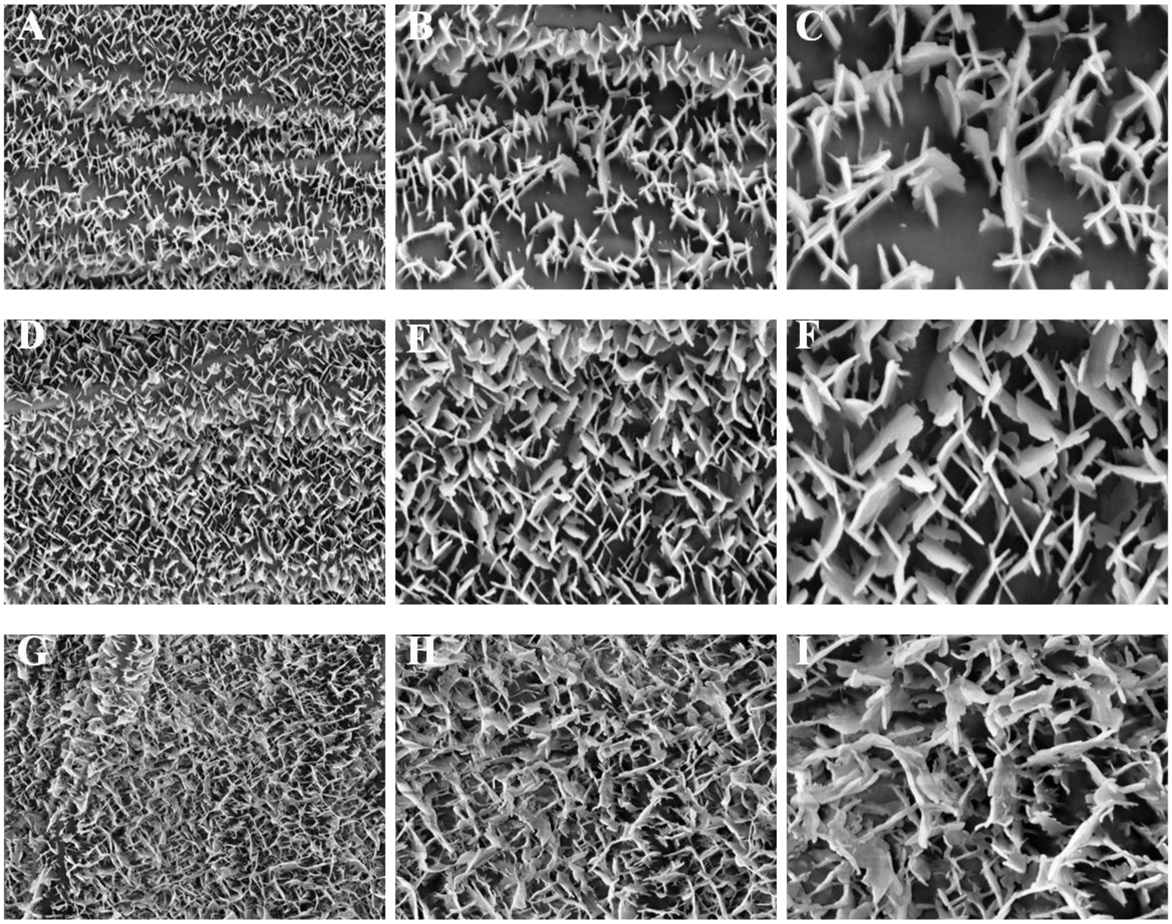

Figure 3.1. Scanning electron micrographs of the creeping bentgrass cuticle layer as influenced by irrigation treatments. . A, B, C: 100%ET treatment at 5,000, 10,000 and 20,000x, respectively. D, E, F: 50% ET treatment at 5,000, 10,000 and 20,000x, respectively. G, H, I: 25% ET treatment at 5,000, 10,000 and 20,000x, respectively.

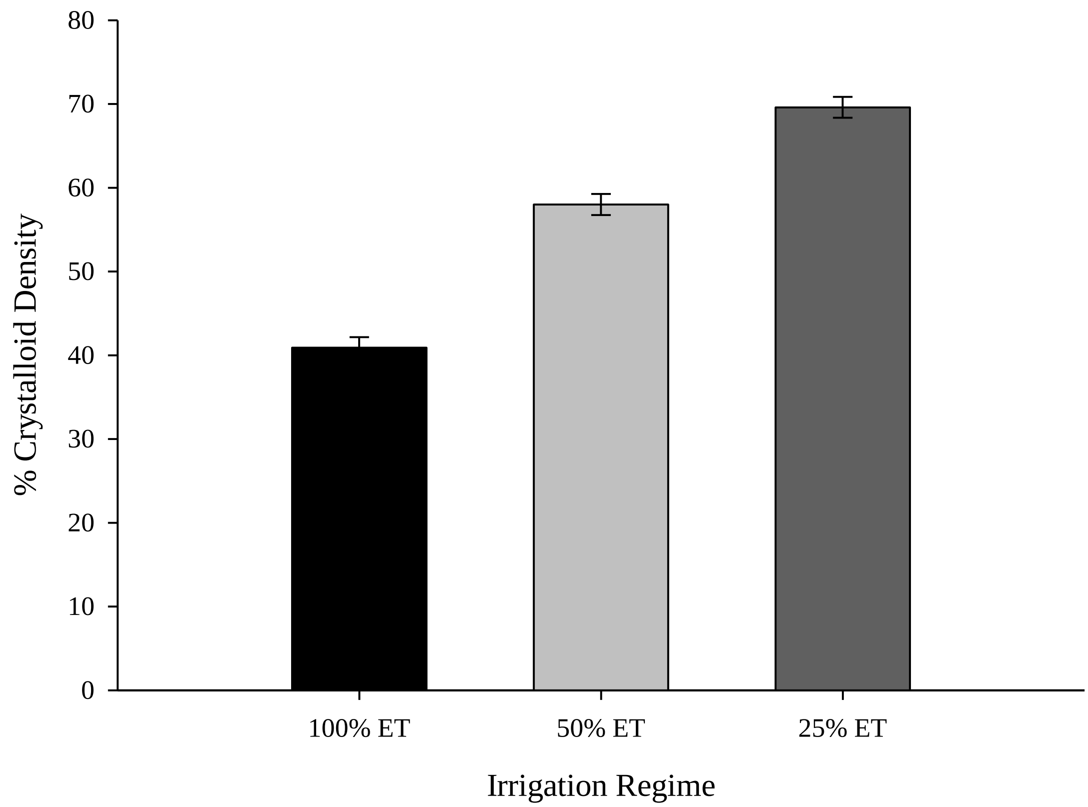

Figure 3.2. Crystalloid density as affected by main effect of irrigation regime. Bars represent mean crystalloid density, with error bars ± the standard error.

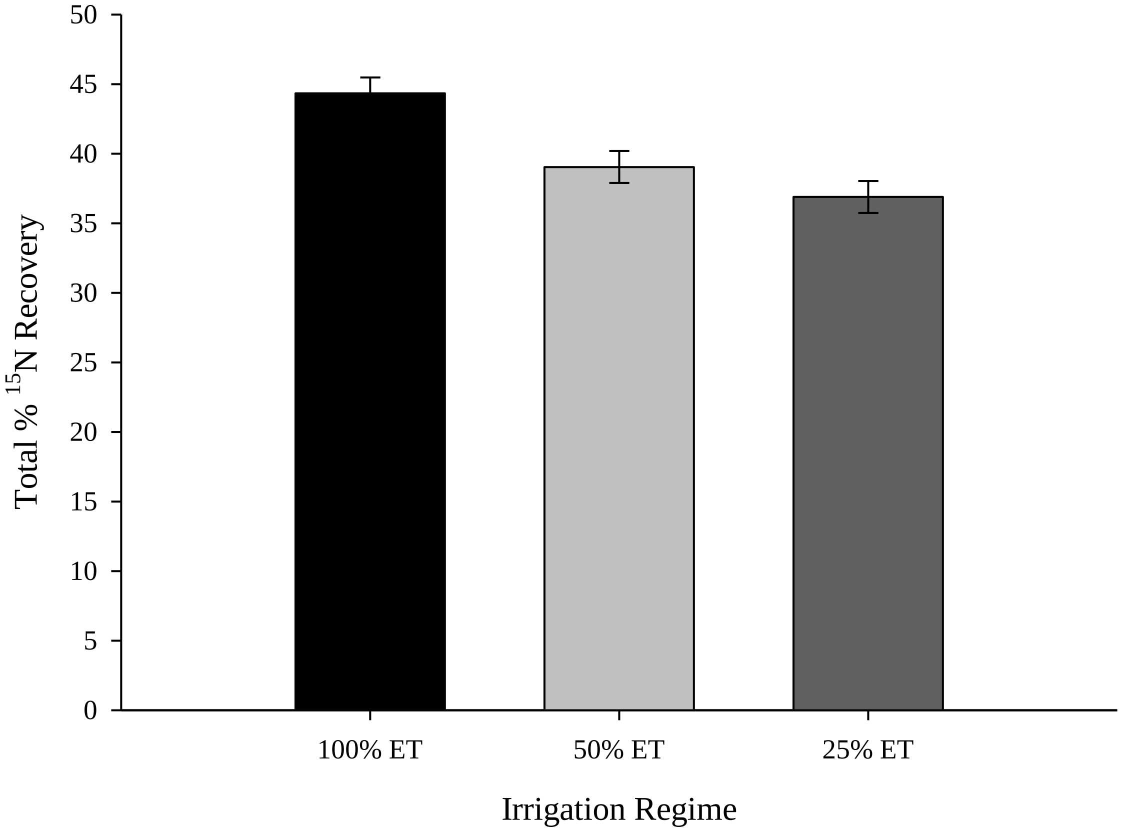

Figure 3.3. Total % ^{15}N recovery as affected by irrigation regime. Bars represent mean crystalloid density, with error bars ± the standard error.

Table 3.1: Analysis of Variance of Total Cuticle Wax and %15N Recovery by the Main Effect of Irrigation: Parameter (group), p-value, comments

Parameter	p-value	(Comments)
Total Cuticle Wax	0.5752	increasing pattern
Alkanes	0.6244	(pattern of increasing amount over
Fatty Acids	0.4495	all wax groups as irrigation treatment
Primary Alcohols	0.5538	reduced)
%15N Total Recovery	0.0011*	decrease as drought increases
Clippings	0.0565	pattern of decreasing with treatment
Roots	0.0207*	less than 1% ^{15}N in roots
Thatch	0.6189	pattern of decreasing with treatment
Crystalloid Density	0.0001*	increases with treatment

*- denotes means significantly different due to irrigation treatment at an $\alpha < 0.05$.

CHAPTER IV

CUTICULAR RESPONSE OF CREEPING BENTGRASS TO DROUGHT AND ITS INFLUENCE ON FOLIAR APPLIED ^{15}N UREA WITH SURFACTANT ADDITION

Introduction

As mention in the previous chapters, drought stress is an ever occurring problem on turfgrass and the future could possibly bring more erratic weather conditions with drought occurrences increasing and becoming more severe. This could continue to provide problems with turfgrass managers unless there is a better understanding of the mechanisms and plant adjustments involved and how they can affect management routines.

The previous chapter demonstrated that as drought stress is induced on creeping bentgrass there are changes that occur to the cuticle layer that affect foliar absorption. The total wax load showed a pattern of increasing as drought becomes more severe. This is from the plant trying to defend itself from increasing water loss through cuticular transpiration. These results are similar to results found in many other plants under drought stress/water deficiencies (Bondada et al., 1996; Kosma et al., 2009; Cameron et al., 2006; Kim et al., 2007a; 2007b; Jenks et al., 2001; Seiler, 1985; Kosma and Jenks 2007; Samdur et al., 2003). Also, it was shown that the morphology of the cuticle begins to adjust to the drought stress and increasing wax. The changes in the cuticle could result in a rougher surface as the epicuticular crystalloids become denser. The accumulation of more wax and change in cuticle morphology creates a barrier that could be less penetrable to foliar absorption. Also, as crystalloids become denser, the solution droplets that are applied to the leaf may have a larger contact angle making the plant less receptive to the foliar solution.

Surfactants have been utilized for many years as an adjuvant to foliar solutions to increase the retention of herbicides in weed control. Research has shown the addition of a surfactant into your herbicide solution, retention and a weed control can increase (Sanyal et al., 2006; Strahan et al., 2000). Surfactants are useful to reduce the surface tension of the solution to allow for a more spreading water droplet and a larger area of contact. This mode of action is intended to increase the amount of solution that touches the intended surface. Nutrient uptake with surfactants has been studied as well. Potassium uptake under field conditions applied to cotton was enhanced with a surfactant solution compared to a water alone solution (Howard and Gwathmey, 1995).

Foliar fertilization is very popular because of its many benefits and possibilities of mixing with other foliar applied chemicals. Also, at times these applications are called for during times of stressful conditions. As shown in the previous chapter, the creeping bentgrass cuticle shows a pattern of increasing under drought conditions, and could reduce the amount of solution that the plant absorbs. Also the morphology of the cuticle changes due to increasing wax and stressful conditions. The effects of drought stress on the cuticle, especially the increase in crystalloid density, could possibly influence foliar absorption and a surfactant addition could increase uptake. A reduction in surface tension could allow the solution to work down through the epicuticular wax crystalloids, and thereby increasing the retention of the solution. For example, crabgrass was shown to be controlled more efficient with a solution that had a surfactant under drought-like conditions (Neal et al., 1990).

The objective of this study was to provide additional evidence of the effects of drought stress on creeping bentgrass cuticle layer morphology and composition. This study also was aimed to investigate if the adverse effects seen by the cuticle under drought stress that hinder foliar absorption can be negated by adding a nonionic surfactant to the foliar applied solution.

Materials and Methodology

Experimental Units

‘A1-A4’ creeping bentgrass putting green plugs were harvested from the nursery green at Thornblade Country Club, Greenville, SC on April 4, 2011. The putting green plugs measured 20.32 cm (8in) in diameter. The nursery green rootzone was an 85:15 (sand:peat) USGA specification mixture. The experimental units for this study were washed thoroughly to remove sand and extra organic matter in the rootzone, and all roots were clipped to approximately 10cm length. Plants were then transplanted into black plastic pots with 85:15 (sand:peat) rootzone mixture and placed in the growth room to acclimate for one month. During the acclimation process, the samples were fertilized bi-weekly with Progressive Turf 10-3-5 liquid fertilizer at a rate of 0.976 g N m^2 , also during the this time the experimental units were mowed three times per week using electrical clippers at approximately 1.27cm with clippings removed. The grass plugs were irrigated daily during the first 5d in the growth room then the irrigation schedule was reduced to three times per week. Once treatments began plants were mowed only once to ensure sufficient plant tissue for the parameters measured. The growth room average conditions during the acclimation time period and experiment were 21^{o}C and 31% RH, with max and min of 23 and 20^{o}C and 71 and 13%RH, respectively.

Irrigation Treatments

Irrigation treatments were a control with 100% ET returned and a drought with 50% ET returned. Irrigation was based off the average daily ET rate of several experimental units. The ET rates were determined gravimetrically by the average mass lost every 24h over a 4d period. Experimental units were fully saturated and pots were allowed to drain for 1h and then the first weight measurement was taken. The next measurements were taken at 24, 48, and 72h after the initial weight measurement. The daily ET rate was calculated by averaging the loss of mass from the previous measurement, and determined to be the average daily ET rate for the experimental units. Experimental units received their respective irrigation for 10d and harvesting of samples took place on the eleventh day.

Cuticle Composition and Quantification Analysis

Cuticle analysis was based off Jenks et al. (1995) with modifications. 1g fresh creeping bentgrass leaf clippings were harvested and submerged in 25ml of hexane for 50s to extract the cuticle layer from the leaves. The extract was then carefully poured into a 25ml glass tube for storage until further preparation. Extracts were then evaporated to dryness under a N_2 stream. The remaining residue was then resuspended in approximately 1ml of hexane and vortexed to remove any residue along the side of the tube. The extract was then transferred into a concial vial and evaporated to dryness again. Cuticle residues were derivatized with bis(trimethylsilyl)-acetimide (BSTFA: Simga-Aldrich) at 100^{o}C for 20min. Silyated samples were analyzed using gas chromatography (GC) with an Agilent Technologies GC 7890A equipped with an Agilent Technologies 5975C Mass Spectrometer (MS). The initial temperature was 50^{o}C held for 2 min, then increased 40^{o}C min^{-1} to 200^{o}C, at which point it began to increase 3^{o}C min^{-1} to 300^{o}C, where it remained for 6min. The GC was equipped with a DB5 MS 30m × 250µm × 0.25µm film column and helium was used as a carrier gas. A sample 2µl was injected, with the injector temperature set at 250^{o}C and a constant flow of 1 µm min^{-1}. Analysis and quantification of peaks were based on Electron Impact Mass Detector (EIMD) scanning from 40-500 atomic mass units (amu), after the detector was turn on at 5min. The quantification of cuticle constituents was based on comparison of area to the surrogate/internal standard tetracosane (10µg) added during sample extraction. Three standards of commonly found chemicals in plant cuticles were separately ran to compare retention times and mass spectra data: an alkane (C_{21}-C_{40}: Simga Aldrich), a primary alcohol (C_{26}: Sigma Aldrich) and a fatty acid (C_{26}: Sigma Aldrich). Identification of cuticle chemical composition was based off standards and spectra in the Wiley NIST library.

Leaf Surface Area Calculation

A surface area of the leaves used for cuticle extraction needed to be determined to report the amount of cuticle per area of leaf tissue. This was measured using an average surface area per weight ratio developed using a WinRhizo software (Regent Instruments, Quebec). Fresh leaf clippings were harvested, weighed and immediately placed on the scanner for analysis. A surface area to weight ratio was developed for each treatment to use for the 1g of fresh leaf tissue in the cuticle extraction process. The measurement was repeated at least 6 times for the two irrigation treatments.

Scanning Electron Microscopy

The creeping bentgrass cuticle morphology was studied using a Hitachi SU6600 Field Emission Scanning Electron Microscope (FESEM) at the Clemson EM Facility in Pendleton, SC. Creeping bentgrass leaves were harvested and fixed with adaixal side up, on aluminum stubs with carbon tape. Leaves were allowed to air dry for 12-24h and sputter coated with platinum before imaging, based on Pathan et al. (2008). Microscope was set at 5kV and a working distance of 10mm. Images were acquired at 2.5, 5, 10, 20, and 30k magnification to analysis and investigation of changes to the cuticle morphology and crystalloids. Images were acquired from areas of relatively flat surfaces and in between vascular tissue.

Crystalloid Density

Methodology was based off Beattie and Marcell (2002) with modifications. SEM images were acquired at 10k × magnification of the creeping bentgrass cuticle. Images were subjected to a uniform adjustment of brightness and contrast before data collection. To determine the % area covered by crystalloids, images were analyzed using NIS Elements (Nikon) and a technique called thresholding and object count. Measurement provided a % area that the crystalloids covered the image.

^{15}N Foliar Absorption

Experimental units received solutions of ^{15}N-labeled urea (ICON Isotopes, NJ) at 0.732 g m^{-2} with or without surfactant addition with a spray chamber at the Clemson University Greenhouse Facility that was calibrated to deliver 561L ha^{-1}. We used a nonionic surfactant (SYNC,Precision Labs.) at 0.125 v/v rate. The application took place at approximately 0800h on the tenth day of treatments, and sampling was conducted 24h after application. The experimental units were divided in half, where half of the tissue went for ^{15}N analysis and half for cuticle extraction and analysis. Analysis of isotopic ^{15}N in tissue samples and fertilizer applied was determined at the University of Illinois at Urbana-Champaign using the automated Rittenburg technique (Mulvaney et al., 1990), on a Nuclide/MAAS 3-60-RMS double mass spectrometer (Nuclide Corporation, Bellefonte, PA).

Statistical Analysis

A completely randomized block design was utilized for this experiment with 6 replications. The experiment was repeated with no significant interaction between runs, so data were pooled. Data were analyzed using JMP 9.0 statistical software (SAS, Cary, NC). Analysis of variance was utilized to determine treatment effects. Means further separated with Student's t test ($\alpha < 0.05$).

Results

Cuticle Analysis and Quantification

The leaf cuticle composition of creeping bentgrass was composed of primary alcohols (~91%), alkanes (<1%), fatty acids (~2.3%), an aldehyde (~3.6%), and a group of unknown compounds (~2.0%). These results confirm the findings in chapter III, that most of the creeping bentgrass cuticle is composed of primary alcohols. Also, the C_{26} primary alcohol, 1-hexacosanol, is the main component in the cuticle comprising approximately 88% of the entire leaf cuticle of creeping bentgrass. The second largest compound is a C_{26} aldehyde, hexacosanal, at ~3.5% of the total cuticle wax. A list of the compounds found in the creeping bentgrass cuticle can be found in Table B.2.

Irrigation treatment significantly affected creeping bentgrass cuticle ($p = 0.027$). The drought treatment (50% ET) significantly increased the total cuticle wax from 2.31μg cm^{-2} to 2.57μg cm^{-2} for the control and drought, respectively (Fig. 4.1, Fig. 4.2). This was an 11% increase in total cuticle wax of creeping bentgrass. The primary alcohols increased significantly from 2.12μg cm^{-2} to 2.37μg cm^{-2} (11% increase) for the control and drought irrigation treatments, respectively ($p = 0.035$) (Fig. 4.3.). Four of the 6 individual compounds in this group increased significantly due to drought irrigation treatment. 1-tetracosanol increased by 23%, 1-hexacosanol increased by 11%, 1-octacosanol increased by 5.9% and 1-triacontanol increased by 43% (Fig. 4.7.).

The fatty acids increased significantly due to the drought irrigation treatment ($p = 0.002$), where the means for control and drought were 0.042μg cm^{-2} and 0.052μg cm^{-2} (25% increase), respectively (Fig. 4.3). There were five individual fatty acids that increased: eicosanoic acid (C20), docosanoic acid (C22), tetracosanoic acid (C24), hexacosanoic acid (C26), and octacosanoic acid (C28) (Figure 4.5). Hexacosanoic acid was the largest fatty acid and increased by 21% from 0.030μg cm^{-2} to 0.036μg cm^{-2} for control and drought respectively. The alkane group only comprises a small amount of the cuticle (<1%), but increased significantly as well ($p = 0.008$). The alkane group showed the largest percent increase of 28%, from 0.015µg cm^{-2} to 0.020µg cm^{-2} for the control and drought, respectively (Fig. 4.3). Although, most of the alkane compounds showed a pattern of increasing only 3 compounds increased in the drought treatment significantly (Fig. 4.6.).

Hexacosanal, (second largest compound) showed no significant changes due to irrigation treatment (Fig. 4.3.). As a group, the unknown compounds did not increase significantly due to irrigation treatment, although the last compound unknown #5 was significantly higher in the drought treatment compared to the control.

Cuticle Morphology

The following description of creeping bentgrass epicuticular waxes follows the terminology and classification from Barthlott et al. (1998) and Jeffree (2006). The adaxial side of the leaves was studied throughout the experiments, though it should be noted the same crystalloid shape was seen on the abaxial side, as well. The crystalloid wax morphologies found on the control treatments will be discussed as the regular wax shape.

The crystalloids found on the surface of the cuticle of creeping bentgrass were primary alcohol plates or membranous platelets. Crystalloids were thin with irregular shaped edges. They ranged from 1-3µm tall and 1-2µm wide. The underlying cuticle layer appeared relatively smooth giving rise to the crystalloids (Fig. 4.4.).

The epicuticular crystalloids of creeping bentgrass were significantly affected by the irrigation treatment. Wax crystalloids appeared to grow to 2-4µm tall and 1-4µm wide due to drought treatment. The shape of the crystalloids remained similar to the control treatments, though the edges of crystalloids appeared to grow and become more irregular. Also, the crystalloids appear to grow and connect with each other or grow around each other.

Irrigation treatment resulted in a significant difference in crystalloid density ($p < 0.001$). The control treatment averaged 43.35% of the area covered by crystalloids, while the drought treatment mean coverage was 59.37%. This was a significant increase of 37% between the control and drought treatments.

<u>^{15}N Foliar Absorption</u>

Foliar absorption of ^{15}N labeled urea fertilizer, expressed as percentage applied, was affected significantly by irrigation ($p = 0.040$) and surfactant ($p = 0.007$) treatments. There was no interaction between irrigation × surfactant treatments ($p = 0.807$).

The irrigation treatment resulted in a 16% decrease absorption in the drought treatment compared to the control. The mean % ^{15}N recovery for control and drought irrigation treatments was 28.91 and 24.92%, respectively. The addition of a surfactant into the solution significantly increased absorption of applied ^{15}N. The mean % ^{15}N absorption increased from 24.37 to 29.47% for the non-surfactant and surfactant treatments, respectively. The data represent approximately 21% increase in percent recovery when a surfactant is added (Table 4.1.).

Total cuticle wax and %^{15}N absorption was evaluated to determine if the amount of cuticle wax influences foliar absorption. The correlation data presented an $r = -0.2396$ for total wax and % ^{15}N absorption with no surfactant addition ($p = 0.274$). The data of total wax and % ^{15}N absorption for the surfactant treatments revealed $r = 0.1125$ (p = 0.609). Neither correlation provided a significant relationship.

Discussion

Creeping bentgrass cuticle layer increases due to reduced water stress, which has been demonstrated in various other plant species (Bondada et al, 1996; Cameron et al., 2006; Kim et al., 2007a; Kosma et al., 2009). Koch and Ensikat (2008) suggested that cuticle wax changes due to environment, and mostly the change is more quantitative rather than qualitative, meaning the change is a response of the cuticle as a whole, not just one compound or group. This type of response was seen with creeping bentgrass in the present study. The cuticle wax groups increased as a whole due to drought stress. Another interesting observation is the major portion of the cuticle that is composed of one single compound, 1-hexacosanol (88%). Steigler (2010) reported similar results of creeping bentgrass wax load sampled from a putting green, which had approximately 90% of the cuticle composed of 1-hexacosanol. The primary alcohols comprise 92% of the total cuticle of the leaves of creeping bentgrass which were covered in epicuticular waxes. Jetter et al. (2006) stated that many Poaceae species have large quantities of their cuticles composed of ß-diketones or primary alcohols which supports these results.

The cuticle morphology plays a significant role in the protection of the plant, and could create more of a barrier to foliar applied solutions, hindering uptake. Yoshimitsu et al., (2002) found that as surface roughness increased the contact angle of a water droplet increased, by reducing adhesion. In the present study we observed surface morphological changes due to drought stress, where wax crystalloids grew and density increased. This is similar to results of relative humidity affecting crystalloid shape in various plant species. Low air humidity was shown to increased wax crystalloid density and wax amount that resulted in decreased wettability (Koch et al., 2006). This is important to the present study because although we were unable to create a method of accurately testing leaf wettability, we did see an increase in crystalloid density, which might be an indicator for reduced wettability and thus reduced foliar absorption.

It has been shown that crystalloid morphology has a direct relationship with chemical composition (Jeffree et al., 1975; Barthlott et al., 1998; Bargel et al., 2006). Jetter et al. (2006) reviewed that high percentages of alcohols in the cuticle could lead to leaves covered in epicuticular wax structures. Jeffree (2006) states that crystalloid shape falls under broad categories and these shapes could be modified slightly by certain chemical compounds that are present in the cuticle. Because of the small modifications found with the crystalloids of creeping bentgrass under drought stress, it could be postulated that the minor chemical composition differences resulted in these morphological changes. Unfortunately, we were unable to determine the difference in composition of crystalloids because our sampling technique included epi- and intra-cuticular wax components.

Foliar absorption was shown to be affected by irrigation treatment. The reduction in foliar absorption could possibly be due to the irrigation treatments affect the cuticle making the leaf surface more hydrophobic. We did not test the wettability of the leaf surface because of the fine leaf texture. Research has shown that there is possibility for uptake of a solution through stomata (Eichert and Goldbach, 2008). Eichert and

Burkhardt, (2001) determined that stomatal aperture and density had an effect on foliar uptake. Stomatal uptake could have been hindered by stomatal closure due to the drought stress resulting in a reduction in foliar uptake as well.

These differences although statistically significant, may not have biological significance because of the small difference between the means. A possible explanation for this is that after foliar application of the nutrient solution, plants were placed back in the growth room, where there was very little air movement and an ideal environment for creeping bentgrass growth and foliar absorption, which allowed the droplets to stay on the surface of the leaf much longer than if plants were located outside. Humidity has been shown to have an extreme impact on foliar absorption, with a higher level of relative humidity increasing foliar uptake of a solution (Ramesy et al., 2005; Schonherr, 2001; Schonherr et al., 2005). External conditions like temperature and internal conditions such as, metabolic activity may have an impact on the absorption of the solution (Gaussoin et al., 2009; Marshner, 1995). The ideal growth room conditions allowed for a great environment for the absorption of the solution because there were no disturbances to the surface that would cause evaporation or movement off of the leaf surface and had an excellent temperature for creeping bentgrass growth. If experiment was conducted outside, the results could have shown a more drastic difference that could have more biological significance.

The use of surfactants has been widely practiced throughout turfgrass and agriculture maintenance. In the present study data suggests that by adding a surfactant to your foliar solution you can increase nitrogen uptake levels for creeping bentgrass.

Sanyal et al. (2006) studied the effects of a non-ionic surfactant with barnyardgrass and green foxtail, and found an increase in the spread of a primisulfuron droplet when a surfactant was added to the solution. In the present study, the addition of the surfactant could have aided in spreading the solution over the crystalloids on the cuticle surface, possibly increasing foliar absorption. Research in peach (*Prunus* persica) with iron revealed that a solution with surfactant reduced surface tension and increased iron concentration in the plant (Fernandez et al., 2006).

Correlation data presented for total cuticle wax load and foliar absorption suggest that as the cuticle wax increases there is a pattern of decreasing foliar absorption. Bondada et al. (1997, 2001) observed a highly significant negative relationship total wax and foliar absorption of applied urea on cotton and citrus leaves. Unlike the cotton and citrus leaf studies, the present study utilized separate samples for cuticle wax extraction and % ^{15}N recovery, which could explain poor correlation. In related research a significant negative relationship was seen between foliar absorption and cuticle wax loads sampled from a creeping bentgrass putting green (Stiegler, 2010). In the present study there was no correlation with wax load and foliar absorption for the units receiving a surfactant solution. This could indicate the surfactant aids in penetrating the solution through the cuticle. However, Fernandez and Eichert (2009) stated it is very complex and there are many mechanisms that could be involved with a surfactant's action; reducing surface tension, solubilizing agrochemicals, preventing crystal formation, and retaining moisture in deposits are a few. It is very possible more than one of the mechanisms listed is involved with the surfactant used and resulting in the increased foliar absorption.

Conclusions

The data present here validate the results in the first study where drought stress significantly increases the total wax of the cuticle in creeping bentgrass. Crystalloid density is also affected by drought stress, possibly resulting in a surface less receptive to foliar applied solutions. Percent ^{15}N-labeled urea was affected by both irrigation and surfactant treatments. Results suggest that when creeping bentgrass is under drought stress by adding a surfactant to the foliar solution absorption levels can increase to non-stressed levels.

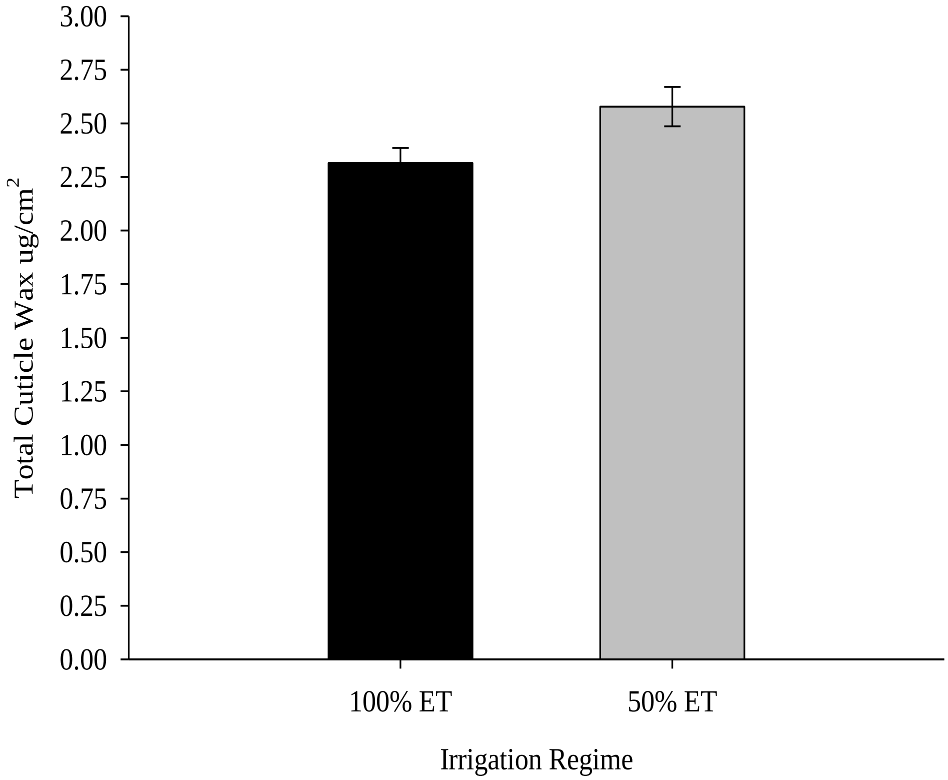

Figure 4.1. Total wax of creeping bentgrass as influenced by irrigation treatment. Expressed in µg of cuticle wax per cm^{-2} of leaf area. Data presented as means ± standard error (SE). Error bars represent SE.

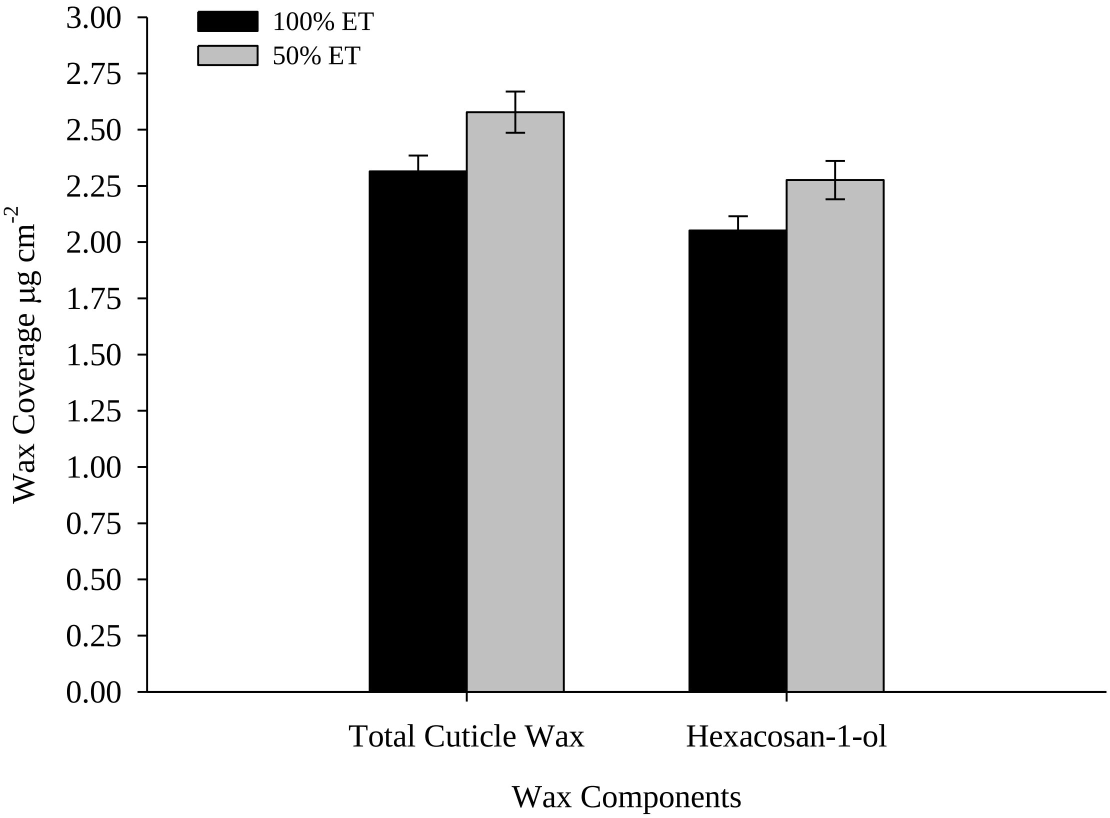

Figure 4.2. Total wax and 1-hexconsanol by irrigation treatment. Data represents means ± standard error (SE). Error bar represents SE.

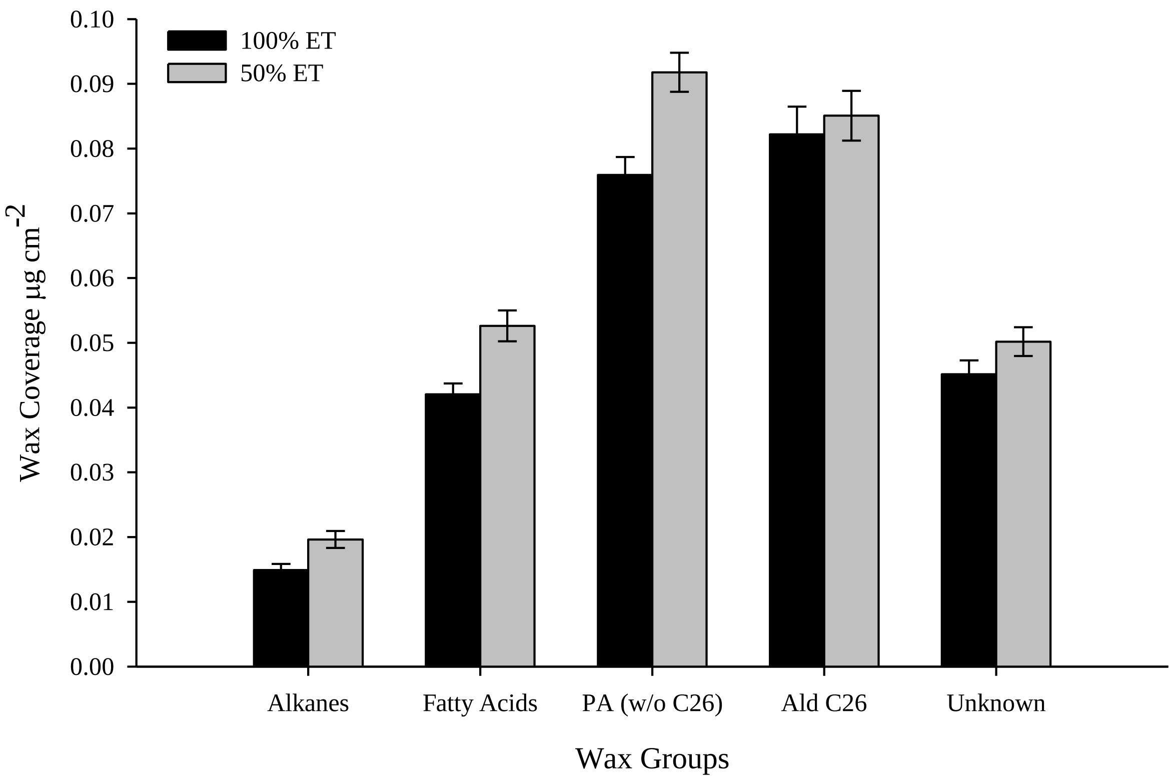

Figure 4.3. Five major wax groups as influenced by irrigation treatment. 1-hexacosanol was removed from the primary alcohol group. Data represents means ± standard error (SE). Error bar represents SE.

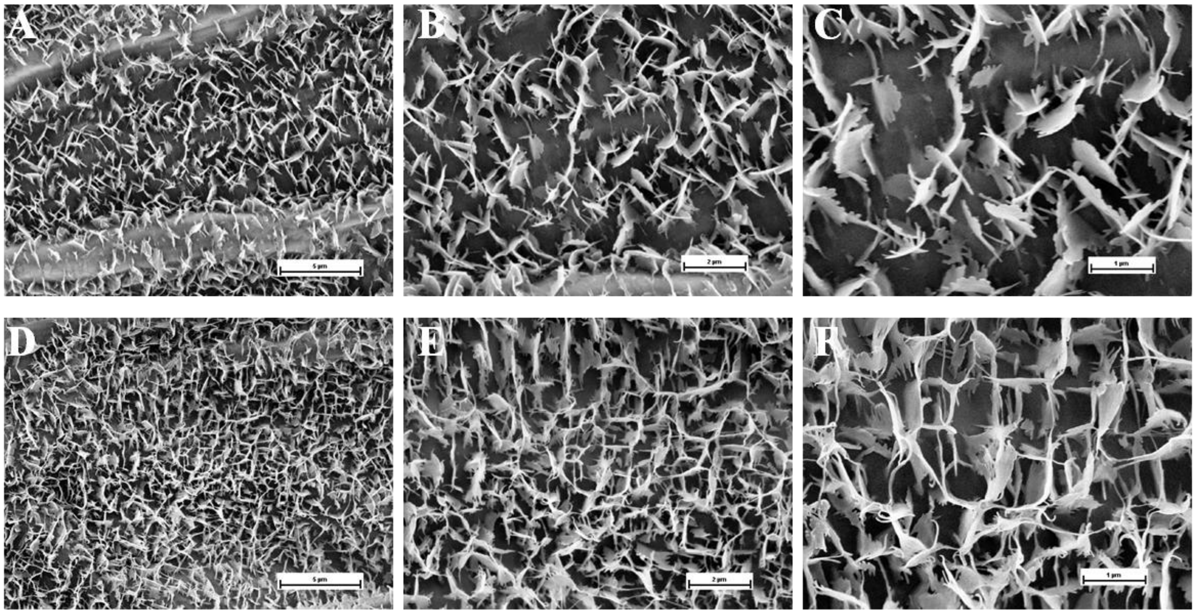

Figure 4.4. Scanning electron micrographs of creeping bentgrass cuticle layer under irrigation regimes. A,B,C: Cuticle of 100% ET treatment at 5k, 10k and 20k x respectively. D,E,F: Cuticle of 50% ET treatment at 5k, 10k and 20k x, respectively. Scale bars are at 5, 3, and 1µm for r 5k, 10k and 20k x, respectively**.**

Table 4.1. Total %^{15}N Recovery for Creeping Bentgrass Clippings as Affected by the Main Effects of Irrigation and Surfactant

Main Effect (treatment)	% Recovery	
Irrigation		
100 % ET	28.91	A
50 % ET	24.92	B
Surfactant		
surfactant	29.47	A
non-surfactant	24.37	B
Irrigation × Surfactant		
100% ET, surfactant	31.19	A
50% ET, surfactant	27.75	A
100% ET, non-surfactant	26.44	AB
50% ET, non-surfactant	22.10	B

Means were separated using Student's t with JMP statistical software.
Means within main effect groups with the same letter are not statistically different ($\alpha < 0.05$).

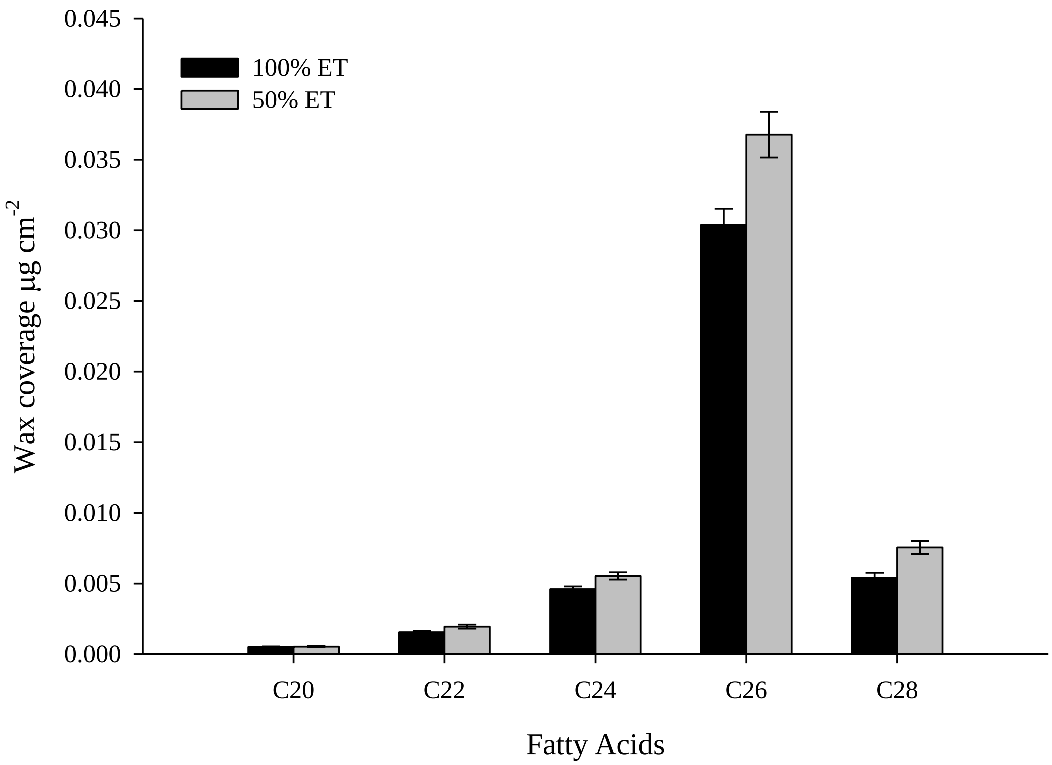

Figure 4.5. Fatty acids in creeping bentgrass cuticle layer as influenced by irrigation treatment. Bars represent means of each constituents of fatty acids found in the cuticle. Error bars represent ± standard error.

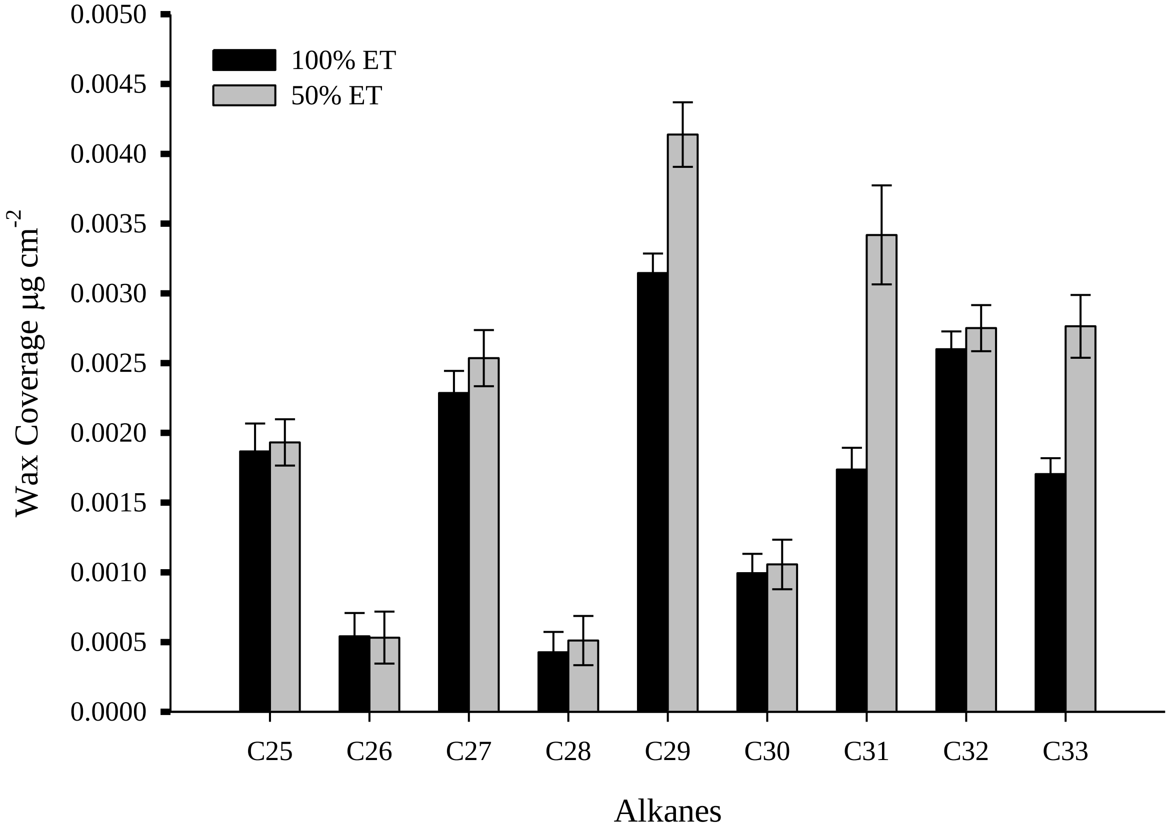

Figure 4.6. Alkanes found in creeping bentgrass cuticle layer as influenced by irrigation treatment. Bars represent means of each constituents of alkanes found in the cuticle. Error bars represent ± standard error.

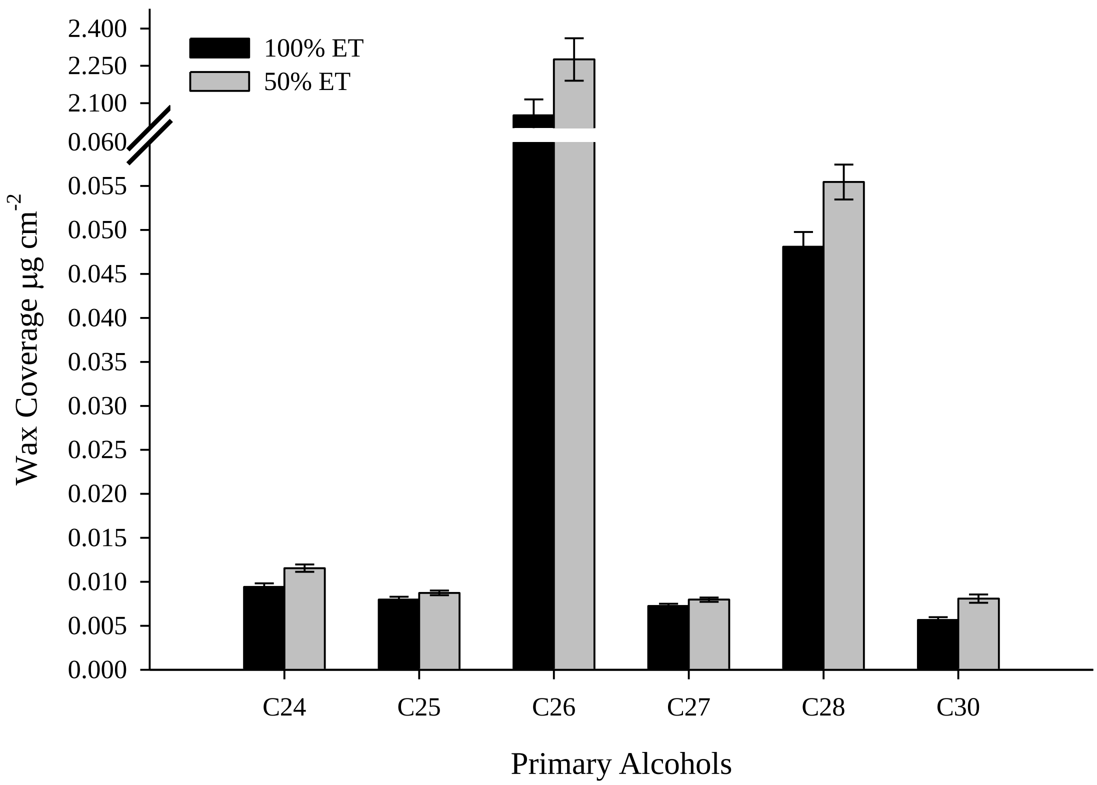

Figure 4.7. Primary alcohols found in creeping bentgrass cuticle layer as influenced by irrigation treatment. Bars represent means of each constituents of primary alcohols found in the cuticle. Error bars represent ± standard error.

CHAPTER V

INVESTIGATION OF MORPHOLOGICAL AND COMPOSITIONAL CHANGES IN AVP1 TG AND WT CREEPING BENTGRASS CUTICLE UNDER DROUGHT STRESS

Introduction

With increasing technologies and bioengineered plants becoming popular in the commercial industry, the need for research to increase our understanding of how these plants will interact with the environment is ever present. Plants that have the ability to tolerant extremes in environmental conditions are a popular research topic. Research recently conducted at Clemson University produced transgenic creeping bentgrass plants that are able to tolerate environmental stresses like salinity and drought (Li et al., 2010). It is important to understand these plants and the mechanisms they utilize to survive in the environment and how they will react under various environmental conditions other.

Transgenic plants allow researchers to develop plants with attributes that can tolerate stress more quickly than traditional plant breeders. For this reason, research is needed to investigate and understand how these TG plants will react and survive under various environmental conditions before released for commercial use. Of particular interest is how plants developed to tolerate and survive under one stress, will react when another stress is present.

As mentioned in previous chapters the cuticle provides the plant with many functions of protection. It is often the case that plants adapted to stressful water stricken environments (arid regions) have a larger, thicker cuticle layer (Shepherd and Griffiths,

2006) providing a level of drought tolerance. Although this is seen in many plants, it is not ubiquitous of all plants that live in water stressed areas. Drought tolerance was increased in transgenic creeping bentgrass by overexpressing a *hva1* gene from barley (Fu et al., 2007).Recent research has shown that an increased cuticle layer could be a marker for drought tolerance. The MYB96 transcription factor in *Arabidopsis* increases cuticle accumulation, data supporting drought resistance through slower cuticular transpiration and chlorophyll extraction was shown in the *myb96-1D* mutant (Seo et al., 2011). Qin et al. (2011) produced a wax-deficient mutant in rice, *OsGL1-1*, that had reduced cuticle deposition, thinner cuticle, increased rate of water loss, as well as, an increase in drought sensitivity. These results demonstrate the cuticle's role in drought tolerance.

A recent focus paper on genetically modified plants discusses how the cuticle can be changed by different adjustments in the genetic material of the plant (Kerstiens et al., 2006). The authors cite various papers with different plant species and genetic changes that produced increases/decreases in wax accumulation and thickness, also cuticle morphology was found to change in many of the genetically engineered plants. Because of the importance of the cuticle in many defenses and a barrier to foliar applied chemicals, it should be studied for changes in genetically modified plants and researched how those cuticular changes could affect how the plant interacts and adapts to its environment.

The AVP1 gene is involved in salinity tolerance and has been shown, in various plants, to increase salinity tolerance when it is overexpressed. Zhao et al. (2006) with rice (*Oryza sativa*), Gao et al. (2006) with tobacco (*Nicotiana* tabacum) and Jha et al (2010) with Arabidopsis have all produced significantly higher salinity tolerance with the over expression of this gene or its homologs. Overexpression of *AVP1* provides enhanced salinity tolerance by increasing the ability of *NHX1* to pump Na^+ into the vacuole and detoxifying the cytoplasm (Jha et al., 2010; Gaxiola et al., 1999). A vacuolar H± translocating pyrophosphatase, *AVP1* works to produce a larger difference in the electrochemical potential of H^+ between the vacuole and cytoplasm. By doing this it increases the movement of Na^+ into the vacuole through a Na^+/H^+ antiporter, such as *NHX1* (Jha et al., 2010). Research has also shown that overexpression of the AVP1 gene can increase drought tolerance in various plant species (Bao et al., 2009; Park et al., 2005; Pasapula et al., 2011). The objectives of this study were to investigate and compare the cuticle morphology and composition of WT and TG creeping bentgrass under drought stress to determine any possible benefits of the TG cuticle compared to the WT.

Materials and Methodology

Experimental Units

The plants used for this study were harvested at the conclusion of a field trial studying the transgenic grass and its salinity tolerance under field conditions. The experiment investigated ‘AVP1’ transgenic creeping bentgrass (TG) and wild-type creeping bentgrass (WT) planted in square foot plots at the Clemson Turfgrass Research Facility. At the conclusion of the field trial, experimental units were harvested from control plots using a 10.7 cm^{-2} cup cutter. Four samples were taken from each square foot plot. The harvested samples were then subjected to a through washing cycle to remove the native soil and organic matter in the rootzone and thatch layer. Once removal of all soil and debris, samples were planted in black plastic pots with 85:15 (sand:peat) USGA specification rootzone mixture and moved into a growth room at Clemson University Greenhouse Facility. The growth room conditions consisted of an average temperature of 21^{o}C with a max and min of 23 and 19^{o}C, respectively. The average relative humidity during the experiment was 61% with a max of 75% and a min of 51%. Experimental units were mowed 3 times per week at 2.54-3.81cm approximately, with electrical clipping sheers. The first week of transplanting, pots were irrigated on a daily basis, then reduced to 3-4 times per week until treatments commenced. Pots were allowed to acclimate to growth room conditions and establish pot for 1 month before treatments began.

<u>Irrigation Treatments</u>

The irrigation treatment consisted of a control and drought, where 100% and 50% respectively, of the ET rate was returned daily. These treatments were conducted daily for 10d and sample harvesting took place on the 11d. Treatments were calculated for both the TG and WT plants.   Evapotranspiration (ET) rates were determined gravimetrically for TG and WT plants, separately. Experimental units were irrigated to fully saturate the soil, after 1h the mass of the pot was taken. 24, 48 and 72h after initial measurement, subsequent mass losses were taken to determine the average amount of water loss daily. The measurements were averaged and calculated to be the average daily ET rates for WT and TG plants separately.

Cuticle Analysis and Quantification

Methods based off Jenks et al., (1995). Two samples weighing 500mg of fresh leaves were harvested from each experimental unit and submerged in 25ml of hexane for 50s to extract the cuticle layer. The extract was then pour into a 25ml test tube and stored for further preparation. The 25ml extract was evaporated down to dryness under a nitrogen stream. Samples residues were re-dissolved in approximately 1ml of hexane and vortexed. The extract was transferred to a conical vial and evaporated again. The derivatizing agent bis(trimethylsilyl)-acetimide (BSTFA: Simga-Aldrch) was added to the residue and heated at 100^{o}C for 20min. Silyated samples were analyzed with an Agilent Technologies GC 7890A Gas Chromatograph equipped with an Agilent Technologies 5975 Mass Spectrometer (MS). The GC had a DM5 MS 30m × 250µm × 0.25µm film column using helium as a carrier gas. Initial temperature was 50^{o}C held for 2min, then increased 40^{o}C min^{-1} to 200^{o}C, then increased 3oC miin^{-1} to 300^{o}C, at which point it remained unchanged for 6min. Injection size was 2µl with a temperature of 250^{o}C and a constant flow of lµm min^{-1}. Analysis of chemical constituents was based on Electron Impact Mass Detector (EIMD) scanning from 40-500 atomic mass units (amu), which was turned on at 5min. Three standards commonly found in plant cuticle waxes were ran separately, an alkane (C_{21}-C_{40}: Simga Aldrich), a primary alcohol (C_{26}: Sigma-Aldrich) and fatty acid (C_{26}: Sigma-Aldrich) to compare spectra and retention times to cuticle chemicals. All quantification of the cuticle were based on comparison of peak area to the surrogate/internal standard tetracosane (10µg) added during sample extraction. Cuticle chemical component identification was based off mass spectra from peaks in samples compared to the standards and spectra in the Wiley NIST Library. Both samples taken were analyzed and the analyses of both were averaged for each experimental unit.

Leaf Surface Area Calculation

To develop a surface area for the leaf tissue used for cuticle extraction WinRhizo software (Regent Instruments, Quebec) was utilized. Leaves were sampled from experimental units and weighed and immediately placed on the scanner, to prevent curling of leaf and an inaccurate measurement. The scanner provided a surface area for the leaves weighed to develop and surface area to weight ratio. This provided a surface area for the 500mg of leaf tissue used for cuticle extraction. Both WT and TG treatments and control and drought treatments were measured separately to account for any treatment affect that may have affected the surface area of the leaves. Each measurement was repeated at least 6 times.

Cuticle Morphology

A Hitachi SU6600 FESEM at Clemson EM Facility, Pendleton SC was utilized for cuticle morphological analysis. Samples were taken and fixed adaxial side up with carbon tape on aluminum stubs. Samples were allowed to air dry for 12-24h based on methodology described by Pathan et al. (2008). Leaves were then sputter coated with platinum before imaging. Microscope was set a 5kV and a working distance of 10mm, and images were acquired at 2.5, 5, 10, 20, and 30k magnification to study and analyze cuticle morphology.

Crystalloid Density

To investigate the difference in crystalloid density between TG and WT and irrigation treatments images acquired at 10k magnification were studied and further analyzed based off methodology by Beattie and Marcell, (2002). Images were adjusted for uniform brightness and contrast and then analyzed using NIS Elements (Nikon). A technique called thresholding and object count was used to determine % of area covered by crystalloids by treatment.

Statistical Analysis

A completely randomized block design was utilized for this experiment with 6 replications. The experiment was repeated with no significant interaction between runs, so data was pooled. Data were analyzed using JMP 9.0 statistical software (SAS, Cary, NC). Analysis of variance was utilized to determine treatment effects. Means further separated with Student's t test ($\alpha < 0.05$).

Results

TG and WT Cuticle Analysis

Cuticle analysis revealed a significant difference between WT and TG cuticles ($p < 0.001$). The WT cuticle ranged from 3.67-5.99μg cm^{-2}, with the TG cuticle at 5.22-7.42μg cm^{-2}. This demonstrates the variation between cuticles but also the larger amount of cuticle found on the TG grass. The main effect of grass type was highly significant; the means were 4.90μg cm^{-2} and 5.99μg cm^{-2} (~22% increase) for the WT and TG, respectively (Fig. 5.1).

30 individual chemical compounds were found in both WT and TG creeping bentgrass cuticle (Table B.4.). The various chemical constituents that comprise the WT cuticle did not change in the TG cuticle, but the amount of individual components were affected by the genetic modification. The largest increase was seen in 1-hexacosanol (C_{26} Primary Alcohol), in which the WT cuticle had an average of 4.31μg cm^{-2} compared to the TG at 5.36μg cm^{-2} (~24% increase) (Fig. 5.2.). This one compound accounts for 88% and 89.5% of the cuticle for the WT and TG, respectively. Because of the large amount of this single compound, the next largest chemical compound in the cuticle accounts for only 3.3% and 2.5% for WT and TG, respectively. That compound is a C_{26}, aldehyde, hexacosanal (Fig. 5.3).

The other individual wax constituents of the cuticle only comprised 1% or less each of the total cuticles. Although these are small constituents many of the compounds significantly increased in the TG cuticle compared to the WT. For the fatty acids four chemicals were significantly higher in the TG cuticle: octadecanoic acid (C_{18}, 33% increase), eicosanoic acid (C_{20}, 48% increase), tetracosanoic acid (C_{24}, 40% increase), and hexacosanoic acid (C_{26}, 16% increase) (Fig. 5.7). For the group of long chain alkanes, two chemicals showed significant differences, dotriacontane (C_{32}, 37% increase), and tritriacontane (C_{33}) where a 31% decrease was found (Fig. 5.8). Three other primary alcohols were found to be significantly different besides 1-hexacosanol. They were 1-pentacosanol (C_{25}, 11% increase), 1-heptacosanol (C_{27}, 7% increase) and 1-triacontanol (C_{30}, 55% decrease) (Fig. 5.9.). There were five compounds unknown with two of them increasing significantly in the TG cuticle, unknown 2 (26.2% increase) and unknown 4 (82% increase).

There were four main groups of chemicals identified in the cuticle, fatty acids, alkanes, primary alcohols and a group of unknown chemicals. Individual compounds were totaled within each group and investigated for group differences. The fatty acid group as a whole resulted in a significant increase of 0.134-0.155μg cm^{-2} (16% increase) for the WT and TG cuticle, respectively ($p = 0.001$). As a total group the long chain alkanes did not significantly change between the WT and TG cuticle ($p = 0.447$). The largest group of the cuticle is the primary alcohols, solely because of the 1-hexacosanol. By removing 1-hexacosanol and combining the other primary alcohols, there is no statistical difference in the group of primary alcohols ($p = 0.567$). The group of unknown compounds increased from WT to TG, 0.072-0.107μg cm^{-2} (49% increase) ($p < 0.001$).

<u>Cuticle Analysis by Irrigation Treatment</u>

The main effect of irrigation did not provide significant differences in total wax load for TG or WT cuticle ($p = 0.113$). Although, the total wax had no statistical difference, both WT and TG cuticles showed a pattern of increasing in response to the drought stress. For the irrigation treatment, the means for WT cuticle total wax were 4.76 and 5.03μg cm^{-2} for control and drought, respectively. For TG cuticle total wax means were 5.88 and 6.09μg cm^{-2} for the control and drought, respectively.

The fatty acid group was the only group that was significantly affected by the irrigation treatment ($p = 0.047$). The drought treatment increased the fatty acid group from 0.154-0.165μg cm^{-2} (7% increase), for the control to drought, respectively. This change was not significant between TG and WT individually. Although, the other three groups did not significantly increases due to the drought treatment, each group revealed the same pattern of increasing due to drought stress. No individual wax component in TG or WT cuticle revealed statistical change due to the irrigation treatment.

Cuticle Morphology

The description of cuticle morphology follows terminology by Bathlott et al. (1998) and Jeffree (2006). There was a morphological difference between the TG and WT crystalloid shape. The crystalloid shape of both looked similar but there were observable differences in shape. Crystalloids ranged from 1-4µm wide and 1-5µm tall. WT crystalloid is thin and longer in height than the TG crystalloids. The WT crystalloid also appeared more serrated and uneven on the edges compared to the TG. TG crystalloids were thicker and more rigid compared to the WT. Also, there was less variation between crystalloids in the TG and the edges were much straighter with little uneven sides (Fig. 5.5. and Fig. 5.6.).

There was a significant effect seen by the irrigation treatment in crystalloid density ($p < 0.001$). The % crystalloid density increased from 44.23 to 63.83% for the WT on the control and drought irrigation treatments, respectively. The TG crystalloid density was 50.61 and 53.21% for the control and drought irrigation treatments, respectively (Fig. 5.4.). This represents the TG cuticle not responding as much to the drought stress as the WT. The treatment of grass type did not provide significant differences in crystalloid density ($p = 0.089$),

Discussion

Transgenic creeping bentgrass cuticle was studied to determine differences in composition, quantity and morphology. Also, changes in the cuticle due to drought stress were studied to determine if the TG cuticle reacts similarly to the WT when the plant is under stress. It has been shown in previous chapters as well as in many other research projects that the cuticle increases due to drought stress. This is thought to be the plant protecting itself from excessive water loss. Previous work demonstrated that cuticle morphology can change under environmental conditions as the plant adjusts to its environment.

The TG creeping bentgrass was altered to express *Arabidopsis* AVP1 gene which is a proton pump in the vacuole, that has been shown to increase salinity and drought tolerance. The major difference noticed in the cuticle of the WT and TG grasses was the significant increased in total wax in the TG cuticle compared to the WT cuticle. The increase in wax load may result in an increase in drought tolerance. It has been studied in genetically modified *Arabidopsis* and rice that increasing or decreasing wax results in changes in the sensitivity to drought stress. Seo et al. (2011) states that MYB96 induces cuticular wax biosynthesis and accumulation which promotes drought resistance in *Arabidopsis.* Qin et al. (2011) produced a wax-deficient mutant rice plant that showed increased sensitivity to drought stress. Periodic drying events were studied with tree tobacco, and found that wax accumulation increased with each drying event which decreased epidermal conductance and possibly increased drought tolerance (Cameron et al., 2006). The increased wax seen in the TG cuticle could provide the plant with significant drought tolerance protecting from excessive water loss compared to the WT cuticle.

The morphology of the cuticle provides the plant with protection from various harmful interactions the plant may encounter. It provides surface roughness and protection from pollutants, scatters and reflects UV-radiation; also a self-cleaning mechanism has been described and attributed to the morphology of the cuticle (Bargel et al., 2006). The cuticle morphology can also influence the inclusion of foliar applied solutes such as foliar fertilizers; evidence to support this is in the previous chapters. Because the morphology is involved in protecting the plant it is of much importance to researchers to understand how this layer is modified and reacts to the environment. In the present study it was shown that the epicuticular crystalloids for the WT and TG plants differed in morphology. Although they were similar in shape, there were differences that might influence how the plant interacts in its environment.

In the two previous chapters, it was demonstrated the cuticle reacted to drought stress and the morphology changes. This change could represent the reason for reduced foliar absorption seen. In the present study, the WT cuticle reacted to drought stress by increasing crystalloid density, while the TG cuticle showed no difference in the drought and control treatments. This could prove beneficial as it may reduce the need for adjuvants and increase absorption of a foliar applied solution when the plant is under drought stress.

Another interesting finding was the difference in crystalloid shape between WT and TG cuticles. The TG crystalloids were wider and less rough than the more variable

WT crystalloids. The similar shape is expected because of the wax chemical composition between the two grasses did not significantly change, only the amount. Both cuticles were comprised of approximately 88% of 1-hexacosanol, and this is the reason for the similar shape of crystalloids. It has been demonstrated that cuticular wax has the ability to self assemble into the crystalloid shapes based on chemical composition of the wax. Platelet type crystalloids, as found in this study, are normally found in cuticles that are dominated by primary alcohols (Bargel et al., 2006; Jeffree, 2006; Koch et al., 2006). This presents additional evidence that primary alcohols give rise to platelet crystalloid structures on the cuticle of plants.

The small morphological changes in the crystalloids between WT and TG cuticles would suggest that there is a difference in chemical composition in the cuticles. The data did not show any significant changes in chemical composition except that the amount of each chemical was different not different chemicals. The data presented suggest that the increase in fatty acid content and the 1-hexacosanol influences the crystalloid by adjusting the width, roughness and variability of each crystalloid. Jeffree (2006) states that various chemicals like alkanes, aldehydes, esters, ketones and fatty acids can modified platelet shape but further research is needed to define the variations and discriminate between chemicals. With the TG cuticle crystalloids less rough and wider, it may provide a more wettable surface which could increase foliar absorption. A cuticle that is more receptive to foliar applied solutions could increase absorption levels and reduce total input. More research is needed to further understand how crystalloid shape and small adjustments in shape affect such practices as foliar absorption.

Conclusions

In the present study TG and WT creeping bentgrass cuticles were studied for chemical composition, quantification, and morphological differences. The results suggest that the TG grass could provide a more drought tolerant plant than the WT because of the increase in total wax load. The morphological data presented suggest that the TG cuticle could be more receptive to foliar applied solutions than the WT because of the less rough surface. Also, drought did not influence crystalloid density for the TG cuticle which could prove beneficial during stressful events that could hinder foliar absorption by changes seen in the WT cuticle. More research is needed to understand how crystalloid shape and total wax load influences regular maintenance practices, also how other environmental stresses influence the cuticle.

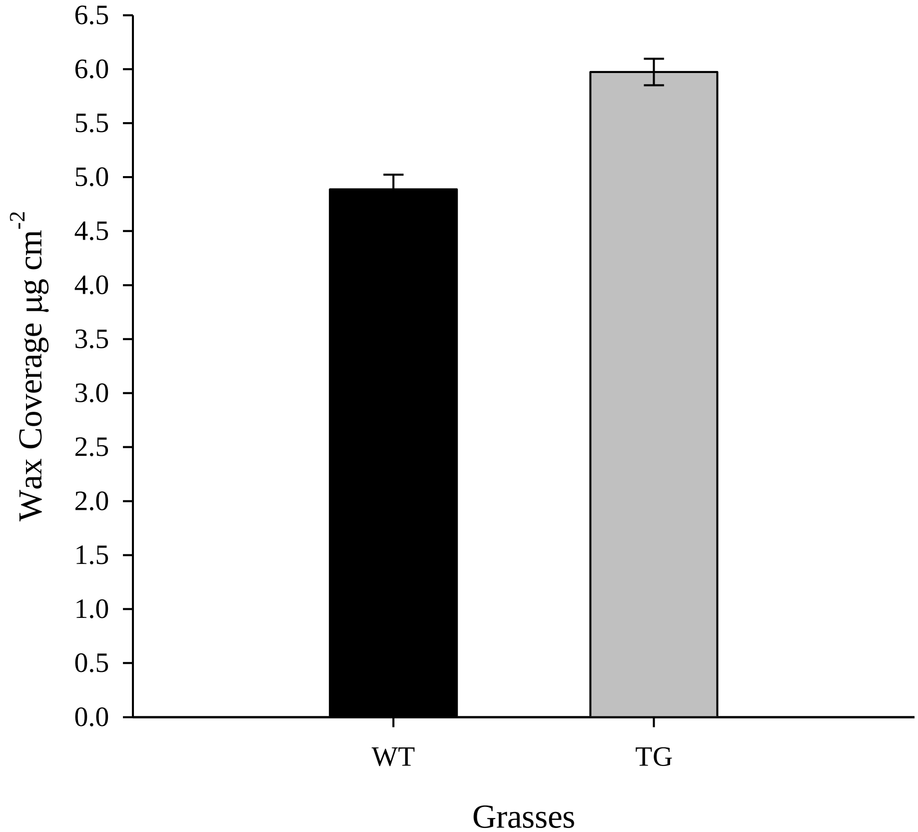

Figure 5.1. Total cuticle wax by WT and TG creeping bentgrass. Expressed in µg cm^{-2}. Data represent means ± standard error (SE). Error bar represents SE.

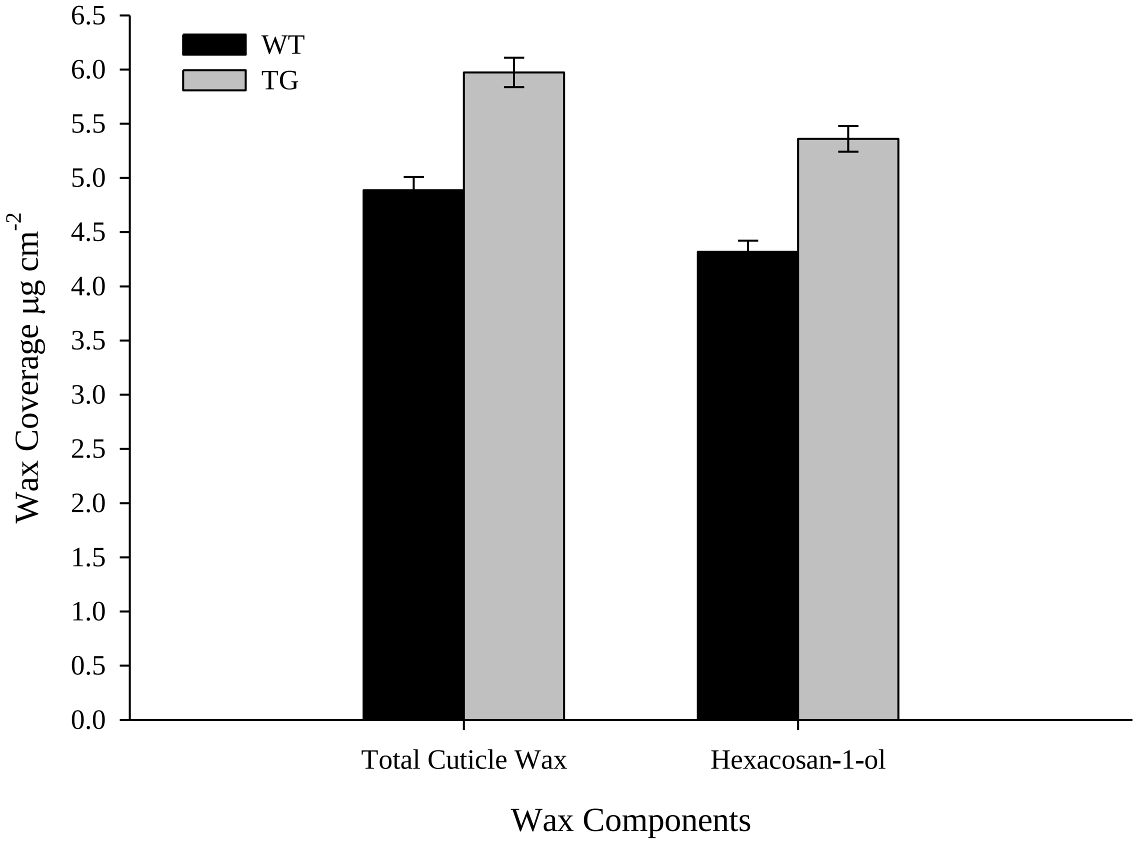

Figure 5.2. Total cuticle wax and 1-hexacosanol by WT and TG creeping bentgrass. Expressed in µg cm^{-2}. Data represent means ± standard error (SE). Error bar represents SE.

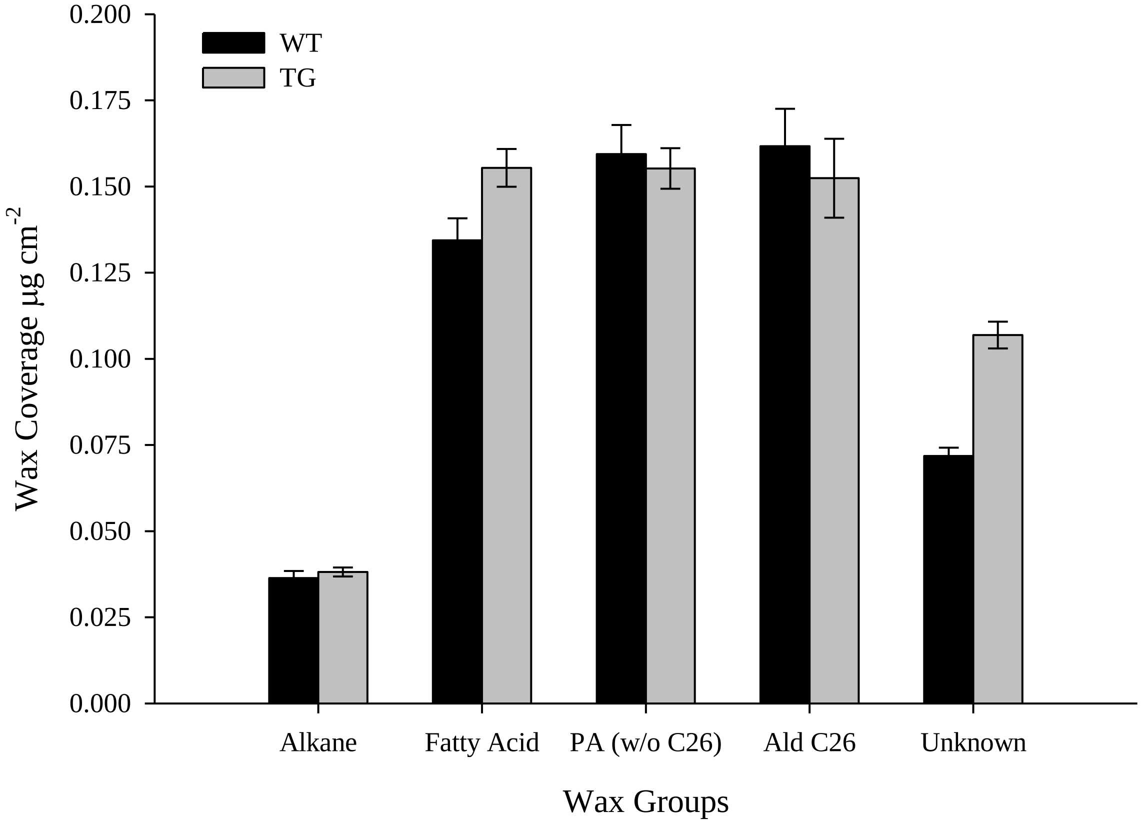

Figure 5.3. Five major wax groups by WT and TG creeping bentgrass cuticle. Expressed in µg cm^{-2}. Data represent means ± standard error (SE). Error bars represent SE.

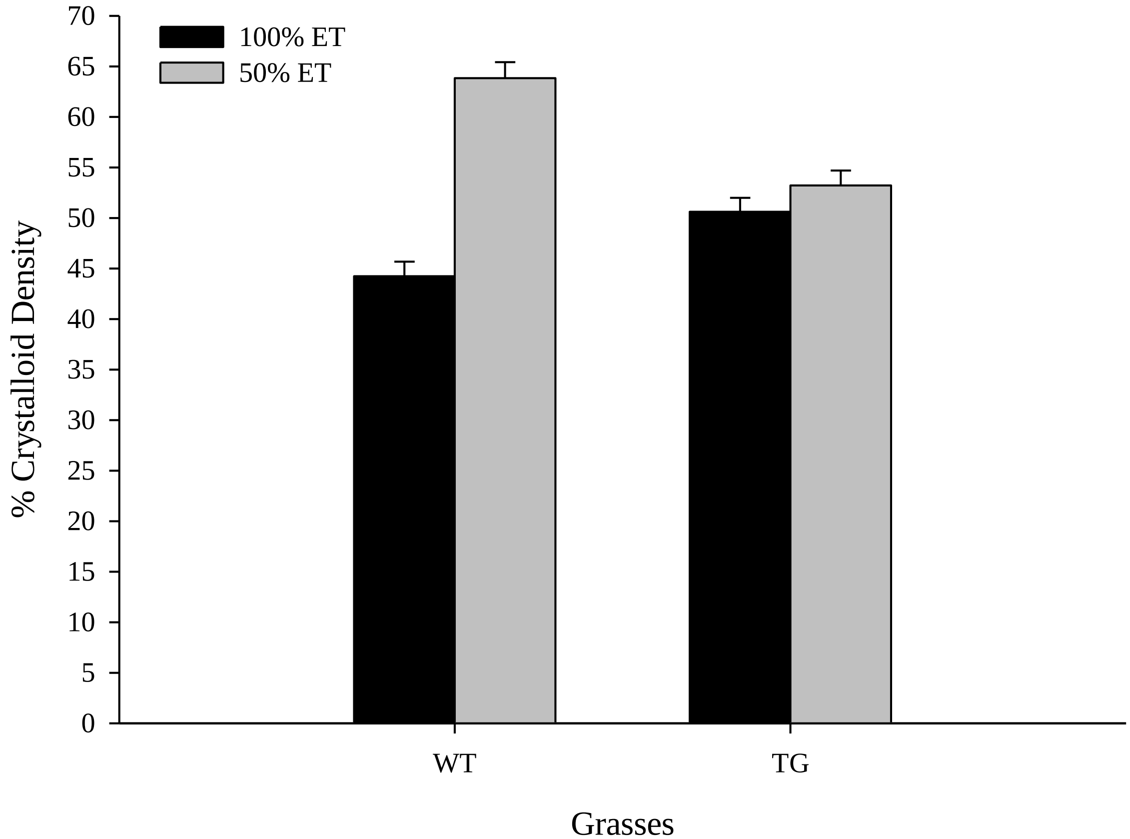

Figure 5.4. Percent crystalloid density coverage as influenced by irrigation treatment for WT and TG creeping bentgrass. Data represent means ± standard error (SE). Error bar represents SE.

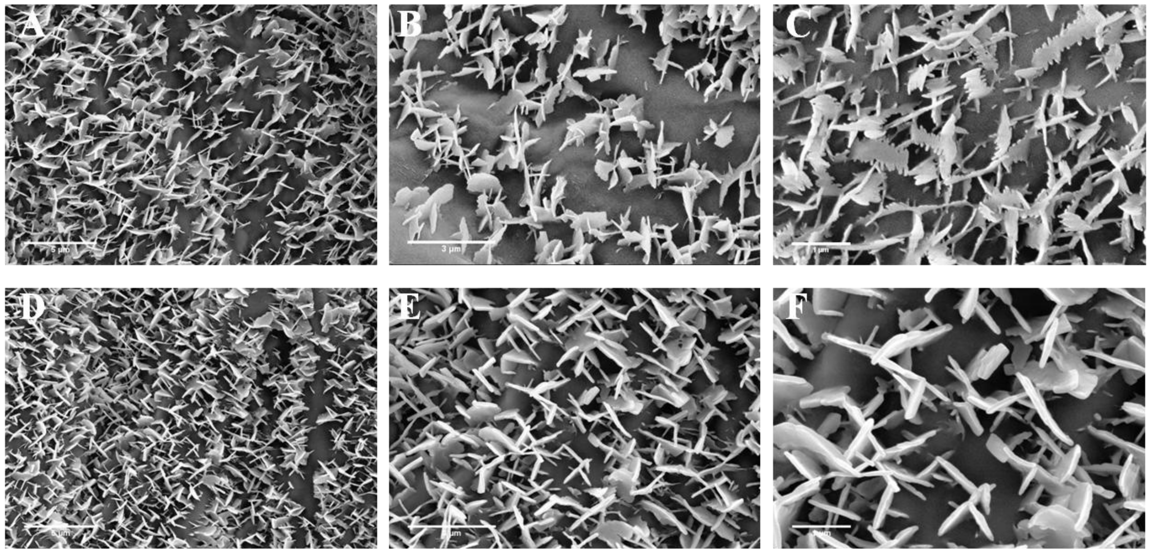

Figure 5.5. Scanning electron micrographs of WT and TG creeping bentgrass cuticle morphology and crystalloid shape under 100% ET treatment. A, B, C: WT cuticle layer at 5k, 10k and 20k x, respectively. D, E, F: TG cuticle layer at 5k, 10k and 20k x, respectively. Scale bars are at 5, 3 and 1µm for images at 5k, 10k and 20k x, respectively.

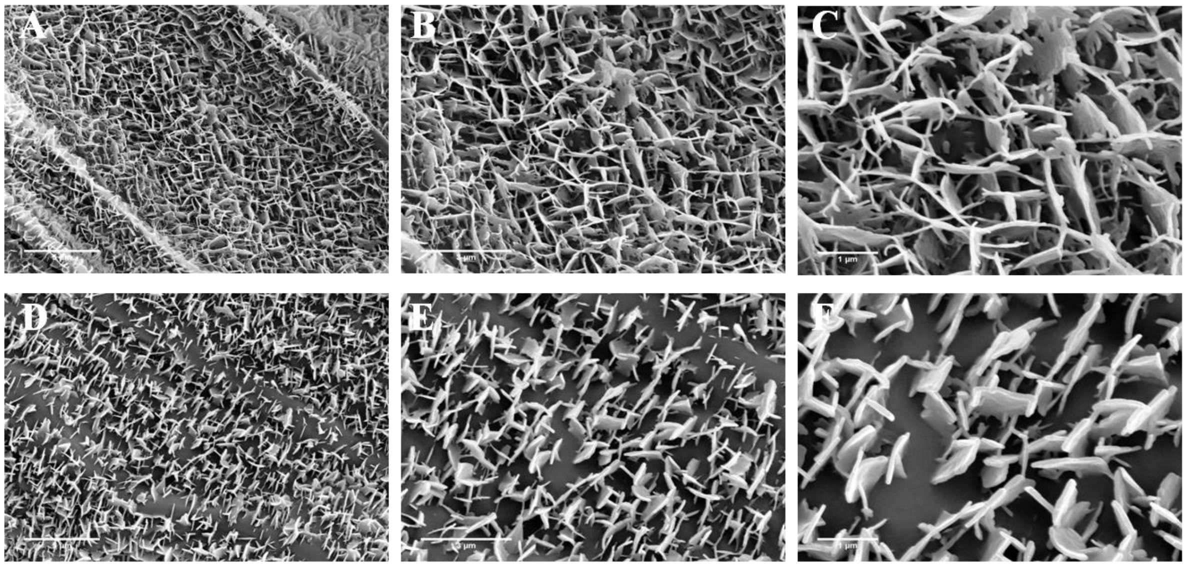

Figure 5.6. Scanning electron micrographs of WT and TG creeping bentgrass cuticle morphology and crystalloid shape under 50% ET treatment. A, B, C: WT cuticle layer at 5k, 10k and 20k x, respectively. D, E, F: TG cuticle layer at 5k, 10k and 20k x, respectively. Scale bars are at 5, 3 and 1µm for images at 5k, 10k and 20k x, respectively**.**

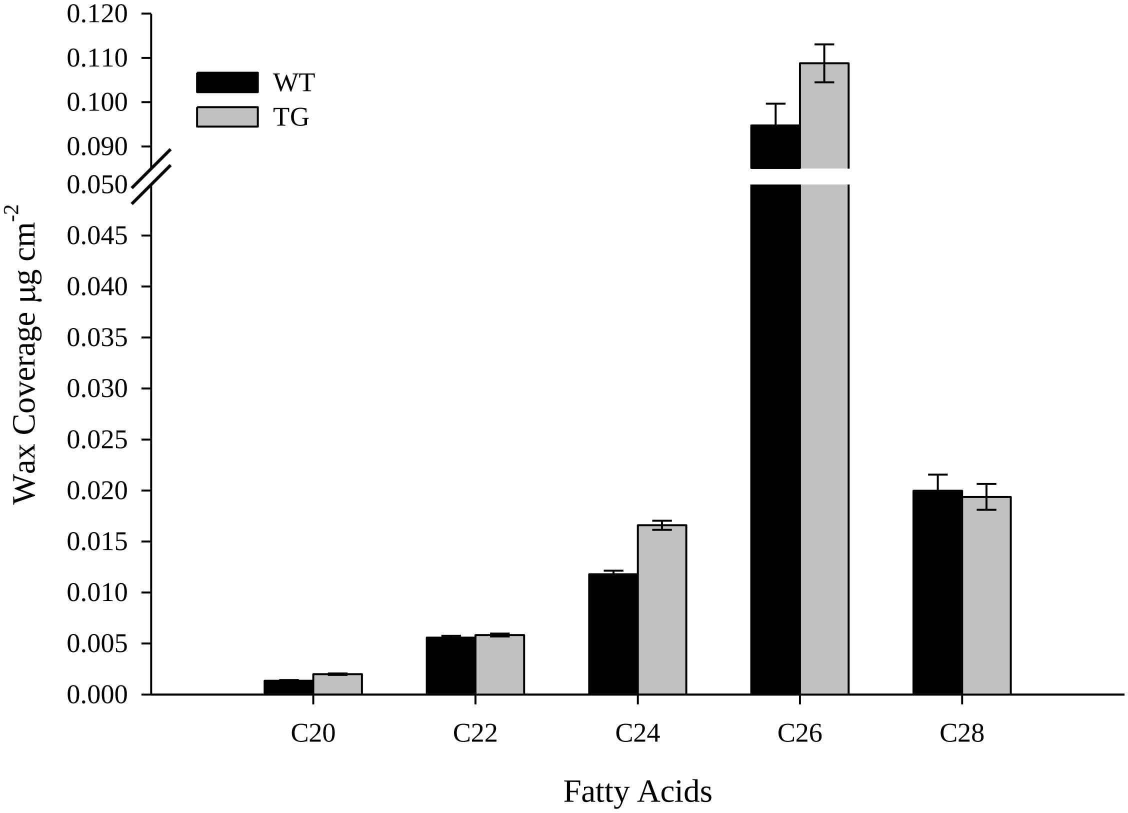

Figure 5.7. Fatty acids found in WT and TG creeping bentgrass cuticle layer. Expressed in µg cm^{-2}. Data represent means ± standard error (SE). Error bars represent SE

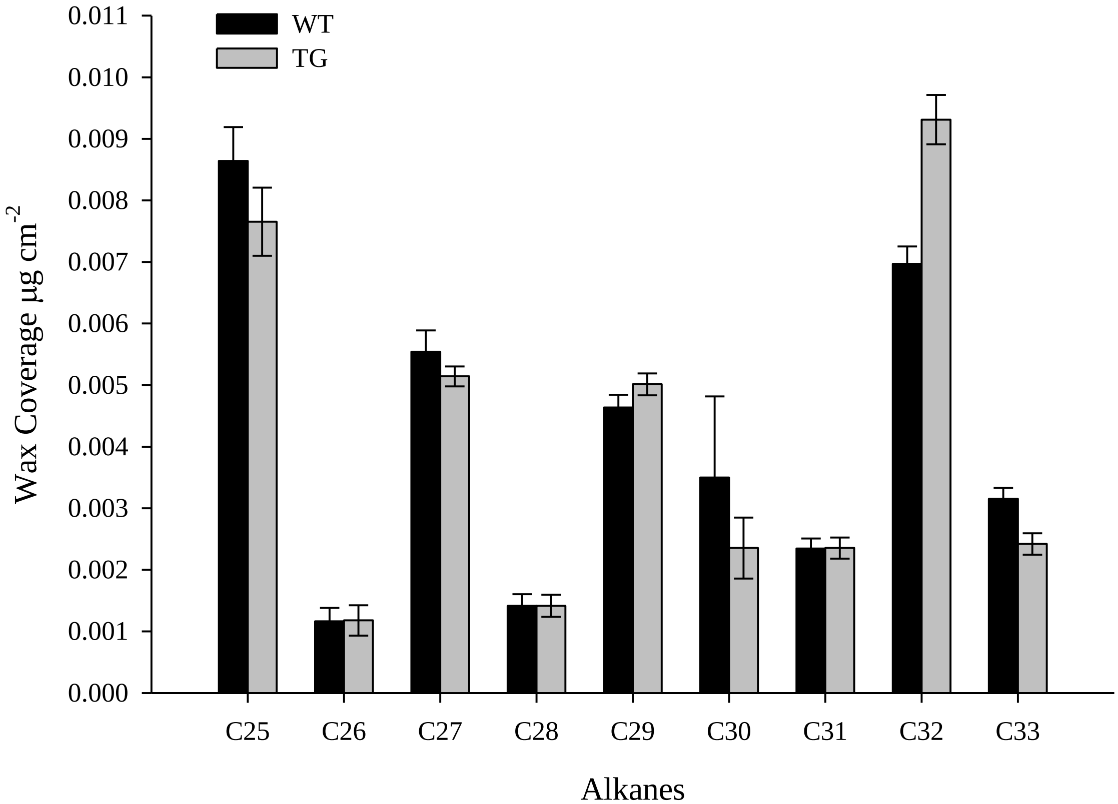

Figure 5.8. Alkanes found in WT and TG creeping bentgrass cuticle layer. Expressed in µg cm^{-2}. Data represent means ± standard error (SE). Error bars represent SE.

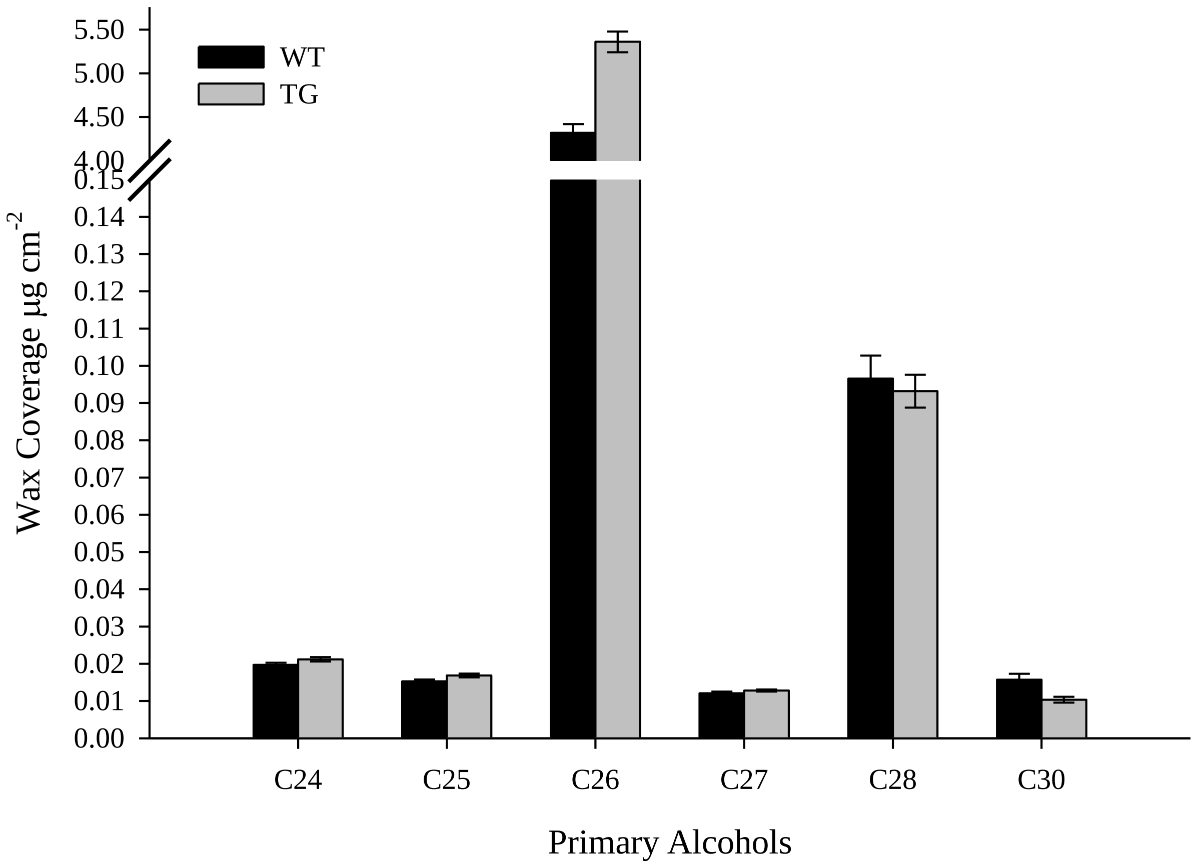

Figure 5.9. Primary alcohols found in WT and TG creeping bentgrass cuticle layer. Expressed in µg cm^{-2}. Data represent means ± standard error (SE). Error bars represent SE.

CHAPTER VI

INVESTIGATION OF WT AND AVP1 TG CREEPING BENTGRASS CUTICLE CRYSTALLOID SHAPES USING FRACTAL ANALYSIS

Introduction

Plant cuticles are receiving a lot of attention currently in material science research, because of the micro- and nano-structures that are produced on their surfaces creating extremely hydrophobic surfaces (Koch et al., 2008; Ramos et al., 2009; Yang et al., 2006). To study these cuticle layers and classify there structure, morphological and compositional analysis has been a popular classification system. Another analysis that is getting quite a lot of attention in surface roughness and hydrophobicity research is fractal analysis which provides the fractal dimension of an object.

Fractal analysis can be used as a classifier to determine mathematically that certain objects that appear to the eye different are statistically different. The fractal dimension not only works as a classifier, but provides information about an object's shape and irregularity. Sztojanov et al. (2009) states that as the fractal object becomes more irregular or convoluted the fractal dimension increases. From this information it can be postulated that as the surface becomes increasing rougher the fractal dimension should increase and provide insight into the nature of the surface.

Plant cuticles are composed mostly of various long chain carbon compounds with other minor wax constituents, which is normally species dependent (Jetter et al, 2006; Bargel et al, 2006, Reiderer and Markstadter, 1996). Also, the morphology that is found on the cuticle is mainly dependent on the chemical makeup of the cuticle layer, and that small changes in the minor constituents could change micro-morphology on the cuticle surface and crystalloid shape (Jeffree, 2006). Unfortunately, there is not enough evidence to classify how each chemical or chemical class affects crystalloid morphology. Previous research has shown that cuticle composition and quantity change due to environmental stress (Bondada, et al., 1996; Kosma, et al., 2009; Cameron et al., 2006; Kim et al., 2007a, 2007b; Jenks et al., 2001; Seiler, 1985; Kosma and Jenks, 2007; Samdur et al., 2003; Koch et al., 2006; Latimer and Severson, 1997).

AVP1 gene has been studied for its assistance in plant salinity and drought tolerance, because of its role in aiding Na^+ sequestering into the vacuole (Jha et al., 2010; Gaxiola et al., 1999). When overexpressed in various plants, AVP1 has increased salinity tolerance over their respective wild-types (Zhao et al., 2006; Gao et al., 2006; Jha et al., 2010). Transgenic creeping bentgrass with overexpression of AVP1 has been produced and showed increased tolerance to salt stress (Li et al., 2010). One interesting thing about TG plants is when a gene of interest is introduced to the plant, other processes and morphological changes occur unexpectedly. For instance, leaf morphology, root length, and dwarf plants are examples of such changes.

In the previous chapter, the cuticle quantity and chemical composition was shown to change significantly from the WT to the TG cuticle. And because cuticle morphology is mostly related to cuticle chemical composition, the difference in crystalloid morphology is expected. The underlying question is why would the cuticle composition be changed by the AVP1 gene and is this change under biological regulation. The objectives of this chapter were to study the cuticle morphology of TG and WT creeping bentgrasses, comparing morphological and chemical differences in the cuticle layers, and to classify the difference using fractal dimension analysis and determine possible reasons for changes in cuticle morphology.

Materials and Methods

Analysis of the fractal dimension of crystalloid shape was done using images acquired from chapter V of this thesis. The same plant material of the WT and TG creeping bentgrass was used as described in the previous chapter. Fractal analysis was determined after investigating results found in Chapter V.

Cuticle Composition

The methodology for cuticle compositional and quantity analysis can be found in the previous chapter (Chapter V).

Images

Images were acquired using a Hitachi SU-6600 FESEM at the Clemson Electron Microscope Facility, Pendleton SC. Samples were taken and fixed on aluminum stubs with two-sided carbon tape and allowed to dry for 12-24h based off Beattie et al. (2006). Samples were sputtered coated with platinum before imaging. Microscope conditions were 10mm working distance, 5kV voltage, under SEM vacuum condition. Images of cuticle morphology and crystalloid shape were acquired at 2k, 5k, 10k and 20k magnification. The 20k images revealed the best resolution for crystalloid morphology differences, so those are the images we sampled from for fractal analysis. Images were subjected to a uniform adjustment in brightness and contrast before analysis.

Fractal Analysis:

Image processing software ImageJ with the Plugin Frac.Lac was used to determine fractal dimension. To sample within images, crystalloids with a good visible edge and away from other crystalloids were chosen for analysis. This provided a good representation of crystalloid shape without other impacting factors. Next, the find edges processing application was applied and then a binary image was created (Figure 6.2 and Figure 6.4.). To only evaluate one crystalloid at a time, a perfect square region of interest (ROI) was drawn around the crystalloid to designate for analysis. Next, the Frac Lac plug-in was utilized to complete the analysis and produce the fractal dimension of each crystalloid using the box-counting process. The box size began at 45% of the picture and decreased down to a size of 2 pixels.

Box-counting equation Frac Lac by ImageJ utilizes:

$$D_B = -\lim (\log N\varepsilon / \log \varepsilon)$$

-where D_B is the slope of the regression line from the log-log of box size (ε) and box count (N) (Frac Lac Manual).

Results

<u>Chemical Composition</u>

The WT and TG creeping bentgrass cuticle layer was analyzed for chemical composition to determine non-target effects the overexpression of AVP1 had on the creeping bentgrass cuticle layer. One of the most interesting results that occurred when studying the AVP1 TG creeping bentgrass cuticle layer was the large increase in total wax compared to the WT cuticle layer. As previously noted, the total cuticular wax increased from 4.90 to 5.99 μg cm^{-2} for the TG to the WT, respectively. This change resulted in a 22% increase from WT total wax load to the TG. The results and data of individual constituents are discussed in detail in the previous chapter. The interesting result to note is that ~95% of the total change in TG compared to the WT cuticle is a result of the change in primary alcohols in the cuticle layer. The other 5% of increase was due to a 2% increase in fatty acids and a 3% increase in the compounds that were unknown.

Crystalloid Morphology

To review results on the difference in crystalloid shape, size and morphology, refer to Chapter V, results, cuticle morphology.

Fractal Analysis

The fractal dimension for the TG and WT crystalloid shape was determined using seven individual crystalloids for each grass sampled from images acquired at 20,000x magnification (Fig. 6.1. and Fig. 6.3). The mean fractal dimension for the WT crystalloid was $Df = 1.51$ with a range of 1.39 and 1.55 for the min and max, respectively. The mean fractal dimension for the TG crystalloid was $Df = 1.19$ with a range of 1.09 and 1.31 min and max, respectively. All fractal dimensions analyzed had a regression line from the log-log plot that had an R-squared value between 0.97-0.99 indicating a very good linear fit for the fractal dimension of crystalloid shapes. The statistical analysis of the fractal dimension of WT and TG crystalloids revealed the crystalloid shape to be extremely different ($p<0.001$) (Fig. 6.5.). This statistic confirms that these shapes were indeed different and there is possibly a process regulating the change in crystalloid shape.

Discussion

A transgenic creeping bentgrass with overexpression of AVP1 gene was studied to investigate morphological and compositional changes in the cuticle layer, and determine fractal analysis on crystalloid shape. The results were utilized to determine and examine non target effects to the cuticle that could be attributed to the genetic modification by overexpression of AVP1.

One of the main results found was the increase in total wax as a result of the genetic modification. This increase that was seen potentially could provide the plant protection by increased drought/salinity resistance. Seo et al. (2011) and Qin et al. (2011) revealed the importance of the cuticle layer as accumulation increases/decreases as the resulting *Arabidopsis* and rice plants show differences in drought resistance and sensitivity. It is possible that the genetic modification of the AVP1 creeping bentgrass selected for salinity tolerance produced a non target effect of increased total wax protecting the plant from potential salinity or drought stress. Research with many different plant species has shown that cuticle wax increases as a result of drought and salinity stress for protecting against increase water loss (Jordan et al., 2001; Bondada, et al., 1996; Kosma, et al., 2009; Cameron et al., 2006; Kim et al., 2007a, 2007b; Jenks et al., 2001; Seiler, 1985; Kosma and Jenks, 2007; Samdur et al., 2003). Since, the AVP1 TG creeping bentgrass cuticle layer total wax is increased significantly from the WT, one possible reason is the that a non target effect increased the total wax preparing itself for salinity or drought stress.

An interesting result to investigate further is the individual wax groups that increased in the TG cuticle compared to the WT. The main increase was with the group of primary alcohols that accounted for ~95% of the total increase in the cuticle of the WT and TG plants. This result is similar to findings in Chapter IV where there was an 11% increase in total wax due to drought stress and ~91% of that increase was due to the group of primary alcohols. Other chemical groups found in the creeping bentgrass cuticle layer show a similar pattern of increasing due to drought stress and the genetic modification of AVP1. For instance, in the present study, the fatty acid and unknown groups accounted for 2.2 and 3.2 % of the increase of the TG cuticle compared to the WT, respectively. The drought study of creeping bentgrass in Chapter IV revealed that the same groups (fatty acid and unknown) accounted for 4.0 and 1.9 % of the increase due to drought stress, respectively (Bethea et al., 2012, unpublished data). Also the group of alkanes in both studies had similar effects, resulting in less than 2% of the total increase in both experiments. This pattern of chemical groups reacting similar to genetic modification and drought stress provides additional evidence that the TG creeping bentgrass is possibly preparing itself for some type of drought/salinity stress.

As previously stated by Jetter (2006), crystalloid shape is mostly dependent on chemical composition, and that minor changes in shape is most likely due to changes in the minor chemical constituents found in the cuticle. Because both cuticles, WT and AVP1 TG, are primarily composed of hexacosan-1-ol, it is expected that the crystalloid morphology be similar in shape. The interesting result is that the platelets on the WT are much more irregular with serrated edges compared to the more rigid and straight edges found on the TG cuticle crystalloid. This can be attributed to the different concentrations of the various constituents found in the cuticle of creeping bentgrass. The minor constituent groups of the WT and TG cuticles, fatty acids and unknown compounds, significantly increased in the TG cuticle compared to the WT. The change in crystalloid morphology could mostly be attributed to the increases seen in the fatty acid group and unknown compound group seen in the genetically modified plants.

The analysis of the fractal dimension of the crystalloid shape of WT and TG cuticle morphology revealed a higher fractal dimension for the WT crystalloid compared to the TG. The WT crystalloid shape had a much more serrated and irregular shape compared to the straighter more rigid shape of the TG crystalloid. The higher fractal dimension was expected because the more convoluted and irregular the shapes the higher the fractal dimension (Sztojanov et al., 2009). This is similar to results by Ramos et al. (2009), where authors found as roughness increases for surfaces the fractal dimension increased as well. Also, Chappard et al. (2003) found that the roughness of titanium was highly correlated with the fractal dimension measured.

The WT crystalloid fractal dimension of 1.51 indicates that it is similar to $1/f$ noise. $1/f$ noise or pink noise is a common phenomenon that is found in nature and many physical processes. This means there is no temporal correlation and the process is at equilibrium (Russ, 1994). The TG crystalloid with a mean fractal dimension of 1.19 suggests it has brown noise and that the processes controlling the crystalloid shape have been altered from the WT and the system has memory (Russ, 1994). These data and the concept of $1/f$ and brown noise indicate the genetic modification in the AVP1 creeping bentgrass caused the changed in the cuticle composition, resulting in the altered crystalloid shape for a specific purpose. The purpose of this alteration is most likely making the leaf surface highly hydrophobic for protection of the plant. Onda et al. (1996) found a fractal dimension of 1.2-1.3 on another wax surface (alkylketene dimer) that resulted in a super-hydrophobic surface, and measured a contact angle of a water droplet to be as large as 174^{o}, which indicates extreme hydrophobicity.

From the results in the present study and Onda et al. it can be theorized that the reason for the change in cuticle composition in the AVP1 cuticle, was to produce an extremely hydrophobic surface. A hydrophobic surface can be beneficial to the plant in many ways. Kosma and Jenks (2007) suggest that plants adapted to salinity stress may have cuticles that repel water more efficiently to reduce and repel saline water droplets from the plant surface and decrease salt ion uptake through leaves. The hydrophobicity of the plant also creates a self-cleaning property can aid to clean off dust and spores but also can protect it by removing microbes and other organisms that could be harmful (Bargel et al., 2006). By the AVP1 creeping bentgrass producing a cuticle that is extremely hydrophobic it's possibly reserving energy that can be used in other protection mechanisms when salinity/drought stress occurs.

Conclusions

In the present study, TG and WT creeping bentgrass cuticle crystalloids were investigated for shape and fractal dimension. It was determine that the AVP1 TG grass had a smaller fractal dimension of ~1.2, and that this indicates an extremely hydrophobic surface. The reason for the change is the genetic modification resulting in salinity tolerance produced a non target effect that changed the cuticle composition therefore changing the crystalloid shape. This altered cuticle could be more efficient at protecting the plant by being extremely hydrophobic and allow other protection mechanisms to work more efficiently.

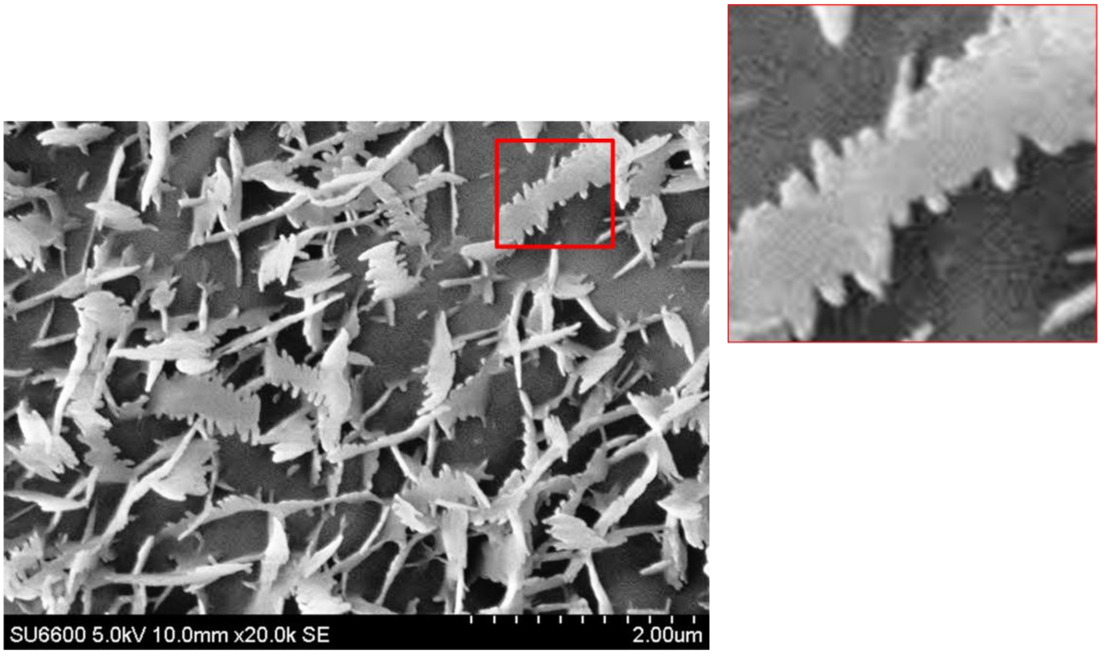

Figure 6.1. Example of WT crystalloid shape at 20,000x magnification. The red box is the sample of the image that was used for fractal analysis of crystalloid shape.

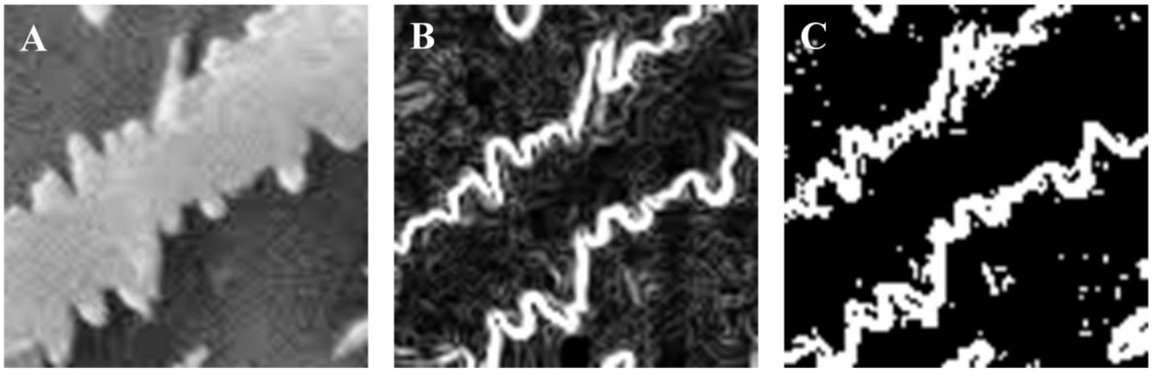

Figure 6.2. Example of the technique used to produce images for analysis using Frac Lac with ImageJ for WT crystalloid. A) Original Sample. B) Image processed using the analysis tool Find Edges and C) The necessary binary image for the Frac Lac determination of the fractal dimension.

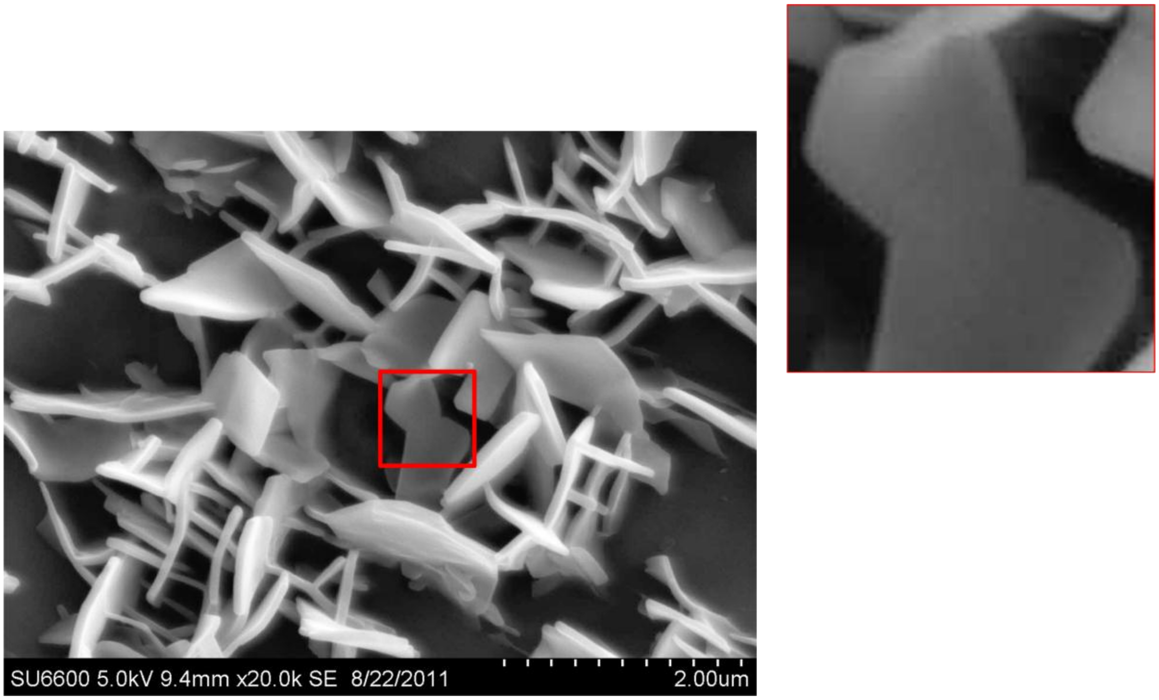

Figure 6.3. Example of TG crystalloid shape at 20,000x magnification. The red box is a sample of the image that was used for fractal analysis of crystalloid shape.

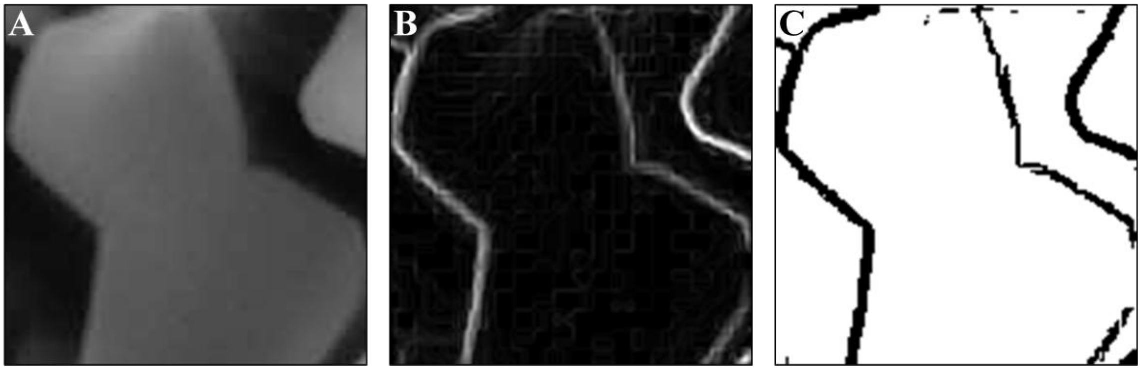

Figure 6.4. Example of the technique used to produce images for analysis using Frac Lac with ImageJ with TG crystalloid. A) Original Sample. B) Image processed using the analysis tool Find Edges and C) The necessary binary image for the Frac Lac determination of the fractal dimension.

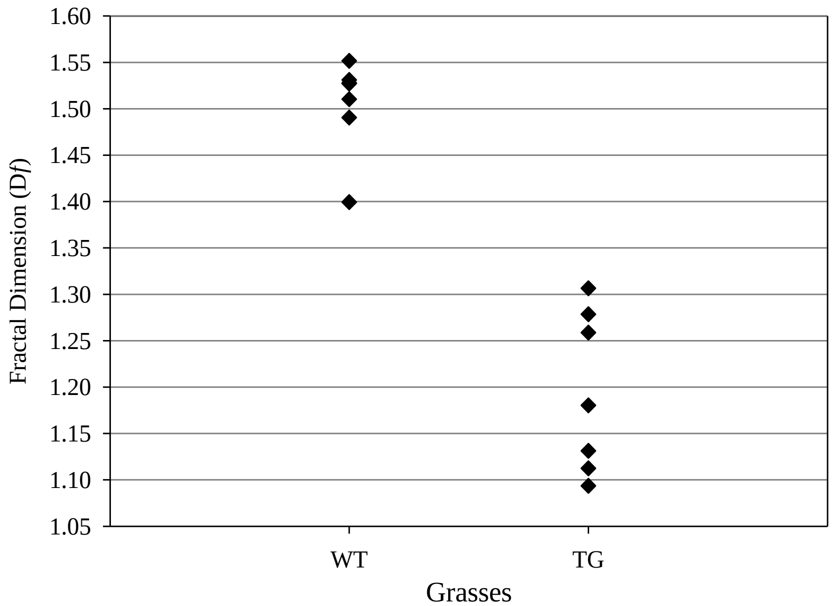

Figure 6.5. Fractal dimension (Df) data points by WT and TG grass. Mean values are 1.50 and 1.19 for WT and TG cuticle crystalloids, respectively.

APPENDICES

APPENDIX A

Cuticle Identification Table

Table A.1. Wax Constituents found in Creeping Bentgrass Cuticle Based on Three Standards: Chemical, Retention Time, Major Mass Spectra Ions

Chemical	Ret. Time	Mass Spec. Fragments	Peak Size (Comments)
Alkanes			
C_{24}	14.7	281.1, 295.1, **338.3**	Internal Standard**
C_{25}	16.5	239.2, 253.1, **352.3**	Medium
C_{26}	18.4	225.2, **281.1**	Small
C_{27}	20.44	281.1, 323.3, **380.3**	Medium
C_{28}	22.4	211.0, **281.1**	Trace
C_{29}	24.45	239.2, 253.0, **281.1**	Small
C_{30}	26.5	221.1, **281.1**	Trace
C_{31}	28.5	207.0, **281.0**	Small
C_{32}	30.8	243.1, 296.3	Small
C_{33}	32.4	281.9, 312.4, **439.4**	Medium
Fatty Acids			
C_{16}	9.74	285.1, **313.3**, 328.3	Medium
C_{18}	12.26	313.1, **341.1**, 356.3	Medium
C_{19}	14.00	239.1, **355.3**	Trace
C_{20}	15.5	**369.3**, 384.3	Medium
C_{22}	19.3	369.3, **397.4**, 412.3	Medium
C_{24}	23.3	397.2, **425.4**, 441.1	Medium
C_{26}	27.3	426.4, **453.4**, 470.4	Big, Known (Standard)**
C_{28}	31.2	453.3, **481.4**, 496.4	Big
Primary Alcohols			
C_{24}	21.5	325.1, 395.3, **411.4**	Big
C_{25}	23.5	409.2, **425.4**	Big
C_{26}	25.64	423.4, **439.4**, 456.4	Very Large, Known (Standard)**
C_{27}	27.5	411.3, 437.4, **453.4**	Medium
C_{28}	29.46	439.4, **467.5**	Big
C_{30}	33.2	439.1, **495.4**	Medium
Terpenoids			
C_{30}	23.0	191.2, 198.0, **206.9**	Small, **Squalene**
Aldehyde			
C_{26}	23.2	334.4, 362.4, 380.4	Large

(Table A.1 Continued)

Table A.1.: Wax Constituents found in Creeping Bentgrass Cuticle Based on Three Standards: Chemical, Retention Time, Major Mass Spectra Ions

Unknowns			
?	28.98	369.4, **401.3**, 437.3	Big
?	29.1	378.3, 439.4, **453.3**	Medium, Spectra and look similar to C_{27} 1^{o} OH
?	29.6	437.4, 468.2, **485.4**	Big (Ketone?)
?	30.3	281.0, 313.1	Small (No Suggestions)
?	33.1	439.2, 495.4	Small (Sterol)

Table showing how peaks were identified. By studying Retention Time vs. Standards and Mass Spectra data peaks were identified as the above labeled chemicals.
Bolded fragment represents the largest fragment in the mass spectra.

Table A.2. Three Wax Constituent Standards: Chemical, Retention Time and Mass Spec. Fragments for Known Standards

Chemical	Ret. Time	Mass Spec. Fragments	Peak Size (Comments)
Alkanes			
C_{21}	10.5	253.2, **296.4**	
C_{22}	11.8	267.1, **310.3**	
C_{23}	13.4	281.1, **324.4**	
C_{24}	15.1	281.1, **338.4**	
C_{25}	16.9	309.3, **352.4**	
C_{26}	18.9	309.2, **366.5**	
C_{27}	20.9	323.2, **380.4**	
C_{28}	23.0	337.1, **394.4**	
C_{29}	25.0	337.3, **408.5**	
C_{30}	27.0	365.3, **422.5**	
C_{31}	29.0	365.3, **436.5**	
C_{32}	31.0	337.3, **355.0**	
C_{33}	32.9	365.4, **407.3**	
C_{34}	34.8	309.3, **326.9**	
Primary Alcohol			
C_{26}	26.0	423.4, **439.5**	
Fatty Acid			
C_{26}	27.8	426.3, **453.4**, 470.4	

* Identification of cuticle chemical composition components was based of studying the spectra and retention times of the known standards. 30 individual components were identified as part of the cuticle of creeping bentgrass, though some were extremely small Bolded fragment represents largest fragment in the mass spectra.

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APPENDIX B

(Additional Tables)

Table B.1. Analysis of Variance of Total Cuticle Wax, %^{15}N Recovery, and Crystalloid Density by the Main Effect of Irrigation: Parameter, p-value, comments

Parameter	p-value	(Comments)
Total Cuticle Wax	0.5752	increasing pattern
Alkanes	0.6244	(pattern of increasing amount over
Fatty Acids	0.4495	all wax groups as irrigation treatment
Primary Alcohols	0.5538	reduced)
%15N Total Recovery	0.0011*	decrease as drought increases
Clippings	0.0565	decreasing as drought increases
Roots	0.0207*	less than 1% ^{15}N in roots
Thatch	0.6189	decreasing with treatment
Crystalloid Density	0.0001*	increases with treatment

*- denotes significant difference by the main effect of Irrigation at an < 0.05.

Table B.2. Analysis of Variance of Wax Constituents Found in Creeping Bentgrass Cuticle as Influenced by Main Effect of Irrigation: Parameter, Chemical Name, p-value, Comments

Parameter	Chemical Name	p-value	(Comments)
Alkanes		0.0085*	increase 28%
C_{24}	tetracosane	-----	Internal Standard**
C_{25}	pentacosane	0.7938	no change
C_{26}	hexacosane	0.9718	no change
C_{27}	heptacosane	0.3402	no change
C_{28}	octacosane	0.7221	no change
C_{29}	nonacosane	0.0003*	increase 31%
C_{30}	tricontane	0.7642	no change
C_{31}	hentriacontane	0.0001*	increase 98%
C_{32}	dotriacontane	0.4348	no change
C_{33}	tritriacontane	0.0001*	increase 62%
Fatty Acids		0.0025*	increase 21%
C_{16}	hexadecanoic acid	0.2767	no change (cutin)
C_{18}	octadecanoic acid	0.0604	no change (cutin)
C_{19}	nonadecanoic acid	0.9912	no change
C_{20}	eicosanoic acid	0.0259*	increase 23%
C_{22}	dococanoic acid	0.0247*	increase 25%
C_{24}	tetracosanoic acid	0.0035*	increase 20%
C_{26}	hexacosanoic acid	0.0025*	increase 21%
C_{28}	octadecanoic acid	0.0007*	increase 39%
Primary Alcohols		0.0353*	increase 11%
C_{24}	tetracosan-1-ol	0.0005*	increase 23%
C_{25}	pentacosan-1-ol	0.0933	no change
C_{26}	hexacosan-1-ol	0.0428*	increase 11%
C_{27}	heptacosan-1-ol	0.1103	no change
C_{28}	octacosan-1-ol	0.0012*	increase 6%
C_{30}	triacontan-1-ol	0.0001*	increase 43%%
Terpenoids			
C_{30}	Squalene	0.6043	no change
Aldehyde			
C_{26}	hexacosanal	0.8377	no change

(Table B.2 Continued)

Table B.2. Analysis of Variance of Wax Constituents Found in Creeping Bentgrass Cuticle as affected by Main Effect of Irrigation: Parameter, Chemical Name, p-value, Comments

Unknowns		0.3508	no change
?	unknown	0.7077	no change
?	unknown	0.2530	no change
?	unknown	0.8058	no change
?	unknown	0.9003	no change
?	unknown	0.0001*	increase 55%

*- denotes significant difference of wax constituents by Irrigation treatment at an < 0.05.

Table B.3. Analysis of Variance of Total Cuticle Wax, %^{15}N Recovery, and Crystalloid Density by the Main Effects of Irrigation and Surfactant: Parameter (treatment), p-value, comments

Parameter	(treatment)	p-value	(Comments)
Total Cuticle Wax	(irrigation)	0.0342*	increase 11%
	(surfactant)	0.5115	no effect
	(irrigation x surfactant)	0.2437	no interaction
%15N Recovery	(irrigation)	0.0403 *	decrease 16%
	(surfactant)	0.0072 *	increase 21%
	(irrigation x surfactant)	0.8079	no interaction
Crystalloid Density	(irrigation)	0.0001*	increase 45%

*- denotes significant difference of the Main Effect Irrigation or Surfactant or Irrigation x Surfactant at an < 0.05.

Table B.4. Analysis of Variance of Wax Constituents Found in WT and TG Creeping Bentgrass Cuticle as affected by Main Effect of Grass: Parameter, Chemical Name, p-value, Comments

Parameter	Chemical Name	p-value	(Comments)
Total Cuticle Wax		0.0001*	increase 22%
Alkanes		0.4467*	increase 5%
C_{24}	tetracosane	-----	Internal Standard**
C_{25}	pentacosane	0.1299	no change
C_{26}	hexacosane	0.8767	no change
C_{27}	heptacosane	0.3450	no change
C_{28}	octacosane	0.8800	no change
C_{29}	nonacosane	0.1278	no change
C_{30}	tricontane	0.3266	no change
C_{31}	hentriacontane	0.9163	no change
C_{32}	dotriacontane	0.0001*	decrease 37%
C_{33}	tritriacontane	0.0040*	decrease 31%
Fatty Acids		0.0001*	increase 15%
C_{16}	hexadecanoic acid	0.2323	no change (cutin)
C_{18}	octadecanoic acid	0.0228*	increase 34% (cutin)
C_{19}	nonadecanoic acid	0.5018	no change
C_{20}	eicosanoic acid	0.0001*	increase 48%
C_{22}	dococanoic acid	0.6702	no change
C_{24}	tetracosanoic acid	0.0001*	increase 41%%
C_{26}	hexacosanoic acid	0.0001*	increase 17%
C_{28}	octadecanoic acid	0.7110	no change
Primary Alcohols		0.0001*	increase 23%
C_{24}	tetracosan-1-ol	0.0799	no change
C_{25}	pentacosan-1-ol	0.0209*	increase 12%
C_{26}	hexacosan-1-ol	0.0001*	increase 24%
C_{27}	heptacosan-1-ol	0.0454*	increase 7%
C_{28}	octacosan-1-ol	0.5574	no change
C_{30}	triacontan-1-ol	0.0001*	decrease 55%
Terpenoids			
C_{30}	Squalene	0.2397	no change
Aldehyde			
C_{26}	hexacosanal	0.2671	no change

(Table B.4. continued)

Table B.4. Analysis of Variance of Wax Constituents Found in WT and TG Creeping Bentgrass Cuticle as affected by Main Effect of Grass: Parameter, Chemical Name, p-value, Comments

Unknowns		0.0001*	increase 49%
?	unknown	0.0016*	increase 26%
?	unknown	0.2239	no change
?	unknown	0.0001*	increase 82%
?	unknown	0.1687	no change
?	unknown	0.5747	no change

*- denotes significant difference between WT and TG constituents of the cuticle layer (cutin)- C_{16} and C_{18} fatty acid are common constituents on the cutin and are not factored into total wax

Figure B.5. Percent ^{15}N Recovery Equations:

$$\text{Fertilizer N Recovered in Sample} = A \times \frac{(S - B)}{(F - B)}$$

A = total N in sample
S = the atom % ^{15}N for the sample
F = the atom % ^{15}N for fertilizer applied
B = the natural atom % ^{15}N abundance (assumed 0.3663)

$$\%\ \text{Recovery of Fertilizer}\ {}^{15}N = 100 \times R / P$$

R = fertilizer N Recovered in Sample (from above equation)
P = amount of ^{15}N Fertilizer applied to area

APPENDIX C

Illustrations

Illustration C.1.Thornblade Country Club, Greenville SC nursery green. Samples for chapters III and IV were acquired at this location.

Illustration C.2. Example of experimental units used for studies conducted in chapter III and IV. Creeping bentgrass pots sampled from Thornblade Country Club and grown in the growth room at Clemson University.

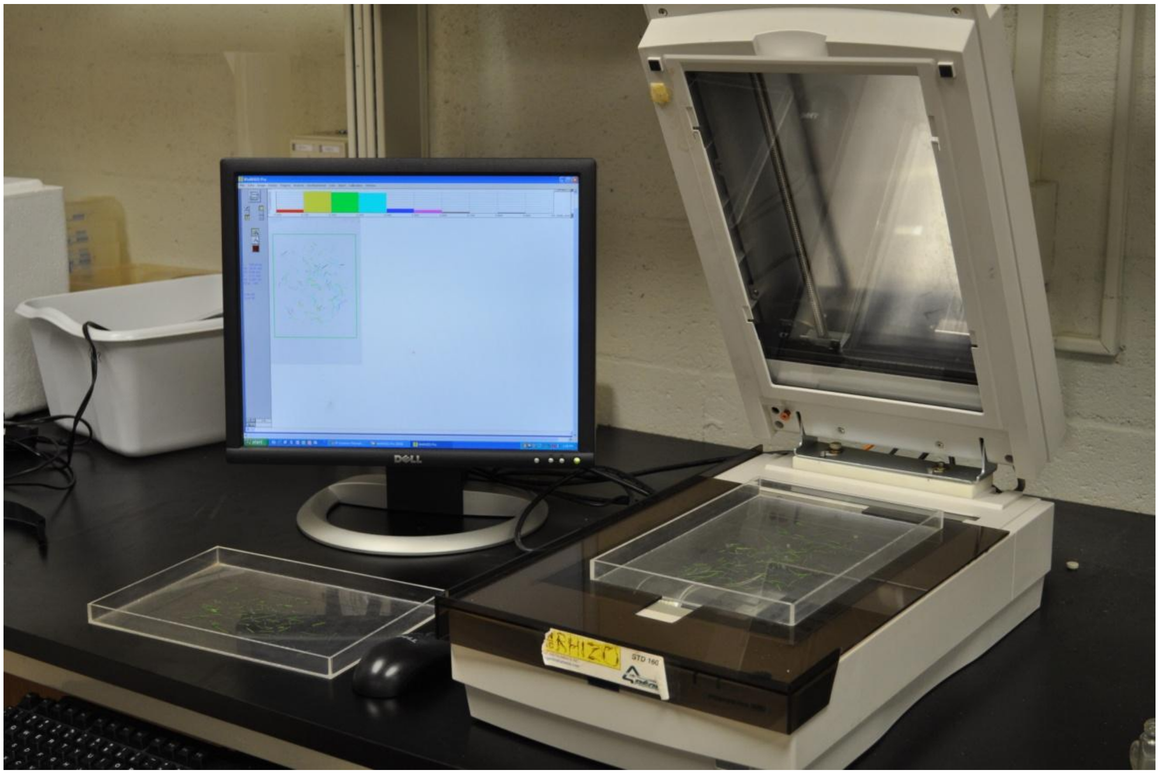

Illustration C.3. WinRhizo scanner set up used for the calculation and determination of leaf surface area. This measurement was used to calculate the surface area of leaf tissue for the 1g of fresh weight used for cuticle extraction.

Illustration C.4. Spray chamber at Clemson University Greenhouse Facility utilized for foliar ^{15}N application.

Illustration C.5. Inside of the spray chamber showing experimental units placed on the stage before ^{15}N application.

Illustration C.6. Cuticle extraction process using 25ml of hexane to remove cuticle and pour into 25ml glass tubes.

Illustration C.7. Example of the 1g of fresh leaf tissue used for cuticle extraction. 25ml of hexane was poured on to each sample and given 50s to extract cuticle before extract was poured off.

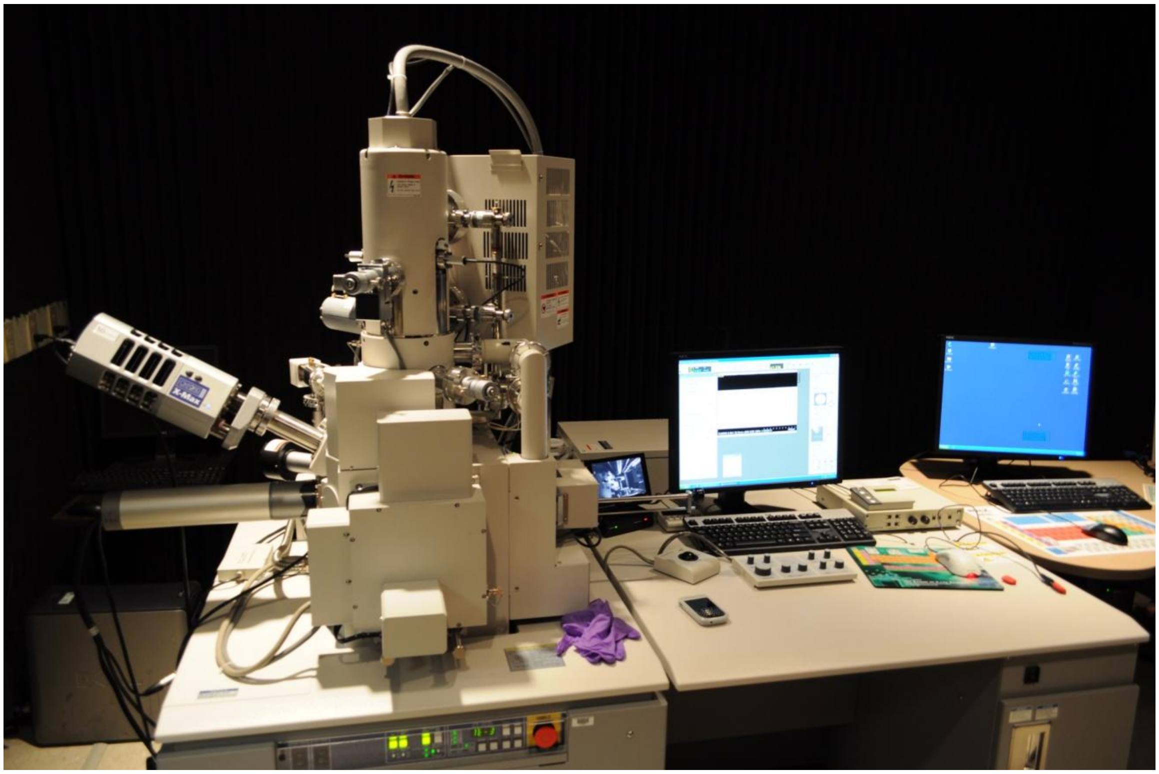

Illustration C.8. Hitachi SU6600 Field Emission Scanning Electron Microscope utilized for investigation of cuticle morphology.

Illustration C.9. Site of transgenic field study. Experimental units for TG drought study were sampled from this site on control plots that received no treatment.

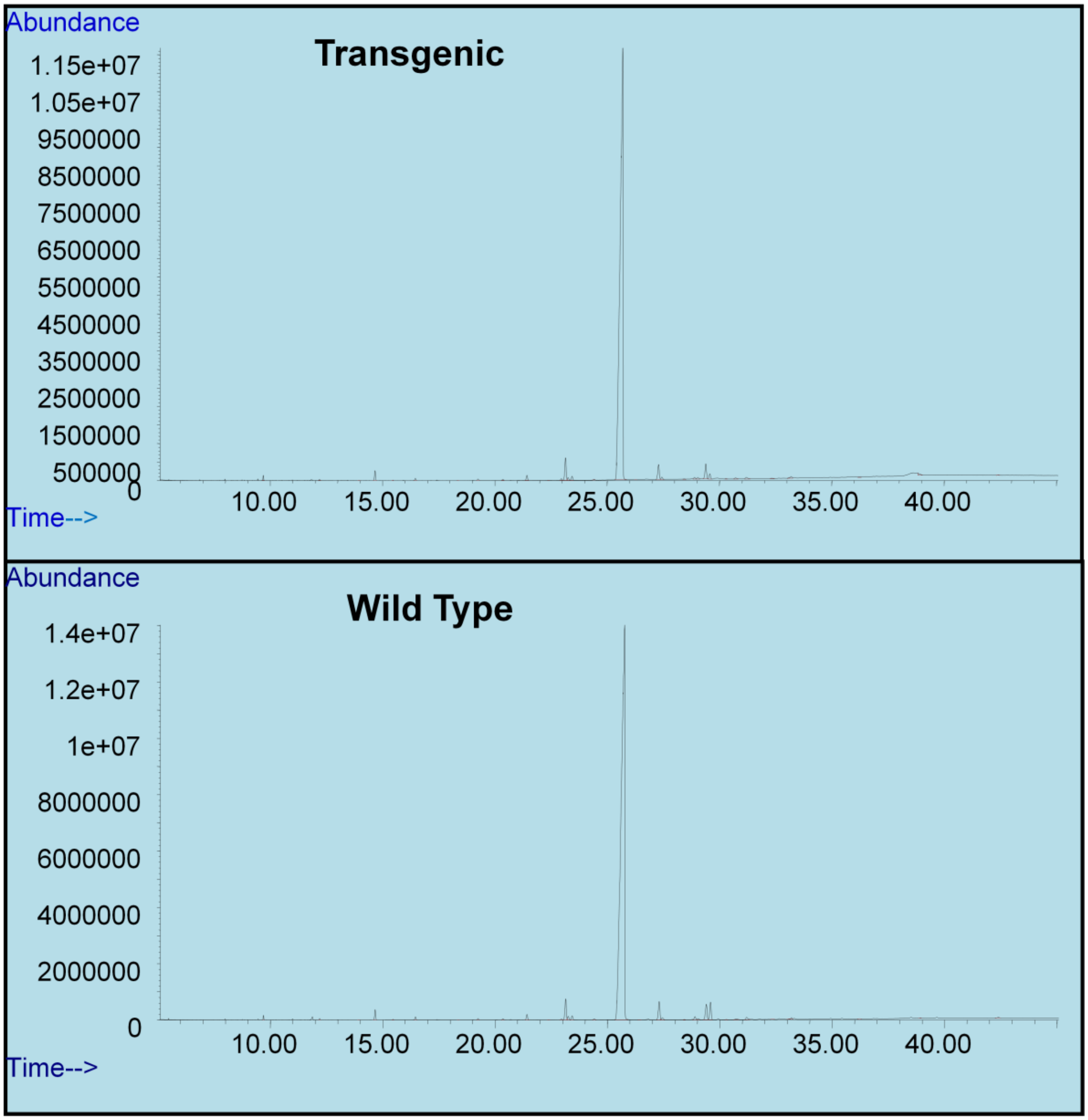

Illustration C. 10. Gas chromatographs of TG and WT creeping bentgrass cuticle layer. Comparing the two reveals the chemical composition of both are similar while the relative abundance of the transgenic cuticle is greater than wild-type.

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